U.PORTO

FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

Biofilm interactions between filamentous fungi and bacteria isolated from drinking water

---

Dissertation for Master degree in Bioengineering

Specialization in Biological Engineering

Faculdade de Engenharia da Universidade do Porto

Ana Filipa Amaral Chaves

Porto, June 2014

Supervisor: Prof. Manuel Simões

Co-supervisor: Dr. Lúcia C. Simões

“Be the change you want to see in the world.”

Mahatma Gandhi

Acknowledgements

Esta dissertação não seria possível sem a participação de vários intervenientes que colaboraram direta e indiretamente, os quais merecem o meu reconhecimento e gratidão.

Primeiramente gostaria de agradecer, ao meu orientador, Professor Manuel Simões, e co-orientadora, Professora Lúcia Chaves Simões, por toda a dedicação, empenho e disponibilidade com que direcionaram e acompanharam esta dissertação.

Ao Departamento de Engenharia Química e ao LEPABE pelas instalações, pelos recursos e pelo financiamento.

Quero agradecer também a todos do Laboratório E-007 por tudo o que me permitiram aprender e pela experiência de poder trabalhar tão de perto com investigadores tão dedicados e profissionais! Um obrigada especial à Inês e à Catarina por toda a paciência e ajuda que me deram ao logo desta etapa! Agradeço ainda à Paula, Sílvia e a Carla do laboratório E-103 por toda a ajuda e disponibilidade.

A 09 e especialmente à Ana Joke Barros, Cabi, Tobi, Sofia, Joana, Diana, Inês por todos os bons momentos que me proporcionaram, por todo o incentivo e amizade que me deram durante estes 5 anos e, no fundo, por tudo o que não se agradece. Ao Tiago que foi uma âncora num ano tão exigente e desafiador. Tenho ainda de agradecer a todos aqueles que se cruzaram comigo durante estes 5 anos e fizeram de mim uma pessoa mais rica e feliz: Guilherme Gordalina, Daniela Saldanha, Mafalda, Maria, Lígia, Alice, Rita, Dulce e Ana Rita esta também é para vós.

À Diana, à Carolina, à Carla e ao Ricardo por me ensinarem que existem amizades para toda a vida!

Acima de tudo gostaria de agradecer à Nanda, ao Pedro, ao Paulo, à Luísa, à Carolina, à Márcia, à São e ao Hugo pelo carinho, amor e apoio incondicional que sempre me deram. Todos vós fazem com que eu consiga ir mais longe do que alguma vez sonhei ou achei possível!

Por fim, esta dissertação é para ti menino Chaves, por me teres sempre ensinado e mostrado que um Chaves nunca desiste…. E um comando também não!

Abstract

The presence of biofilms in drinking water distribution system (DWDS) constitutes one of the currently recognized hazards affecting the microbiological quality of drinking water (DW). Also, biofilms can alter the taste, odour and the visual appearance of water, which is an indication of poor DW quality and may lead to a number of unwanted effects on the quality of the distributed water.

Very few reports on filamentous fungal biofilms can be found in the literature mainly because these fungi cannot fit completely within the biofilm definitions that are usually proposed for bacteria. Nevertheless, fungi are microorganisms that due to their absorptive nutrition mode, secretion of extracellular enzymes to digest complex molecules, and apical hyphal growth are excellent for surface growth. Hence, fungi are excellent candidates for biofilm formation even though this aspect is still poorly understood.

In several environments bacteria and fungi co-habit and interact with each other. These interactions have a wide range of applications and influence both species creating sometimes an increased resistance to antibiotics, antifungal agents and disinfectants.

The aim of this dissertation was to achieve a better understanding of single and dual-species biofilms comprised of both filamentous fungi (ff) and bacteria isolated from water distribution systems. In order to study adhesion and biofilm formation, assays were performed in 96-wells polystyrene microtiter plates. The adhered cells and biofilms were evaluated using crystal violet (CV) dye for biomass quantification and resazurin for metabolic activity (viability) determination. The results showed that the association between both *Penicillum expansum* and *Penicillium brevicompactum* and *Acinetobacter calcoaceticus* in a dual-species biofilm, does not seem to present any great disadvantage for either species and no antagonism can be perceived for biofilms (24 and 48 h). Additionally, the physico-chemical characterization of the microbial surface by contact angle measurements was also performed and indicated that *A. calcoaceticus* is a hydrophilic bacterium and *P. brevicompactum* spores are hydrophobic. Results obtained for *P. expansum* spores applying a novel technique showed that this structure is hydrophilic, which is not the case. However, fungal surfaces were characterized for both fungi as being hydrophobic. Theoretical values of free energy of adhesion of the microorganism with polystyrene surfaces were also

assessed and showed a feasible adhesion for both fungal spores. However, this free energy of adhesion value underestimated microbial adhesion to the mentioned surface.

Disinfection with sodium hypochlorite (SHC) was performed for 48 h single and dual-species biofilms. The biofilms before and after treatment with SHC were analysed using CV dye for biomass quantification and resazurin for metabolic activity quantification. The results obtained showed that biofilms of *P. brevicompactum* are extremely resistant to disinfection when compared with single species biofilms of *P. expansum* and dual-species biofilms of *P. brevicompactum* - *A. calcoaceticus*. Furthermore, the association of *A. calcoaceticus* with both fungi seems beneficial, since the dual-species biofilms are more resistant to disinfection. Additionally, as expected, high inactivation and removal of the biofilms usually occurred when high concentrations of SHC were used. However, total biofilm control was not achieved.

Resumo

A presença de biofilmes em sistemas de distribuição de água é, hoje em dia, um dos perigos reconhecidos quando se fala da qualidade microbiana da água. É de notar que a presença de biofilmes pode alterar o seu sabor, odor e aspecto visual, sendo assim um indicador da sua má qualidade.

Atualmente existem muito poucos dados referentes a biofilmes de fungos filamentosos. Tal deve-se principalmente ao facto destes fungos não encaixarem completamente nas definições existentes de biofilme, já que estas são normalmente propostas para bactérias. No entanto, e devido ao seu modo de nutrição (absorção), à sua capacidade de secretarem enzimas extracelulares capazes de digerirem moléculas complexas e, ainda, devido ao seu modo de crescimento apical nas hifas, os fungos são excelentes candidatos para a formação de biofilmes.

Em vários ambientes, fungos e bactérias coabitam e interagem dando assim origem a um variado leque de aplicações que vão influenciar ambas as espécies. Estas interações podem mesmo levar a um aumento da resistência a antibióticos, agentes antifúngicos e desinfectantes.

Esta dissertação tem como objectivo o melhor entendimento de biofilmes simples e mistos de fungos filamentosos e uma bactéria isolados de sistemas de distribuição de água.

De modo a estudar tanto os fenómenos de adesão como a formação de biofilmes, foram usadas microplacas de 96 poços de poliestireno. As células aderidas e os biofilmes formados foram quantificados através da coloração com cristal violeta (CV) e a sua atividade metabólica (viabilidade) foi avaliada através do uso da resazurina. Os resultados obtidos indicam maioritariamente que a associação entre os fungos *Penicillium expansum* ou *Penicillium brevicompactum* e a bactéria *Acinetobacter calcoaceticus* em biofilmes mistos não apresenta grandes desvantagens e não é possível encontrar relações antagónicas após 24 e 48 horas. A caracterização físico-química da superfície dos microrganismos através da medição de ângulos de contacto mostrou que *A. calcoaceticus* é uma bactéria hidrofílica. Usando uma nova técnica desenvolvida, foi ainda possível caracterizar a hidrofobicidade dos esporos fúngicos, o que indicou que os de *P. brevicompactum* são hidrofóbicos enquanto que os resultados obtidos para os esporos de *P. expansum* indicam que estes são hidrofílicos. No entanto, ambas as superfícies fúngicas foram caracterizadas como hidrofóbicas. Foram ainda obtidos

valores teóricos para energia livre de adesão dos microrganismos à superfícies de poliestireno. Os resultados indicam que para ambos os esporos fúngicos a adesão é possível, o que se verificou experimentalmente. No que toca aos valores teóricos para a energia livre de adesão da bactéria estes indicam que a adesão não é possível o que na realidade não se verificou.

Biofilmes simples e mistos com 48 horas foram ainda sujeitos a tratamentos de desinfecção usando hipoclorito de sódio. Novamente, os biofilmes sujeitos a tratamento e os não tratados foram analisados usando CV e resazurina. Os resultados obtidos mostram que biofilmes simples de *P. brevicompactum* são extremamente resistentes à desinfeção quando comparados com o biofilmes simples de *P. expansum* e biofilmes mistos de *P. brevicompactum* e *A. calcoaceticus*. A associação da bactéria com ambos os fungos aparenta ser benéfica já que os biofilmes mistos são mais resistentes à desinfecção. É ainda de salientar que, tal como esperado, para grandes concentrações de desinfectante a inativação e remoção de biofilmes são mais elevadas.

Contents

Figures List

Tables List

Glossary

BFIs- Bacteria- fungi interactions

CV- Crystal violet

DBP- Disinfection by-products

DLVO- Derjaguin- Landau- Verwey - Overbeek

DW- Drinking water

DWDS- Drinking water distribution systems

EPS- Extracellular polymeric substances

ff- Filamentous fungi

O.D.- Optical density

PS- polystyrene

SHC- Sodium hypochlorite

WHO- World health organization

XDLVO- Extended Derjaguin- Landau- Verwey - Overbeek

Chapter 1

1. Work outline

1.1. Background and project presentation

The supply of clean drinking water (DW) is a very important public health milestone (Berry et al. 2006). Hence, there are many regulations with pre-established parameters (including microbiological ones) in order to ensure that the water provided is safe and free of hazards for the human health.

Drinking water distribution systems (DWDS) are known to harbour biofilms, even if in the presence of a disinfectant. These biofilms are constituted by a microbial community adapted to conditions of low nutrient concentration and high chlorine levels (Simões & Simões 2013). The presence of biofilms in DWDS constitutes one of the currently recognized hazards affecting the microbiological quality of DW and may lead to a number of unwanted effects on the quality of the distributed water. Furthermore, biofilms can constitute a reservoir of pathogenic microorganisms.

Very few reports on filamentous fungal biofilms can be found in the literature, probably because these microorganisms cannot fit completely within restrictive biofilm definitions based on bacteria. However, fungi are especially adapted for growth on surfaces, as evidenced by their absorptive nutrition mode, their secretion of extracellular enzymes to digest complex molecules and apical hyphal growth (Jones 1994). Fungi are therefore excellent candidates for biofilm formation but this aspect is still poorly understood.

It should be noticed that DW quality depends on the specific composition and activity of microbial populations present during the treatment, storage and distribution phases. Therefore, microbial contamination is also a problem for DWDS (Farkas et al. 2012). So, the creation of more robust and adequate disinfection treatments for DWDS is of crucial significance.

1.2. Main objectives

The main objectives of this study is to analyse the ability of selected filamentous fungi (ff) (*P. brevicompactum* and *P. expansum*), commonly detected in DWDS, to

adhere and form biofilms alone or associated with bacteria *A. calcoaceticus*, also isolated from DWDS.

Moreover, some of the possible factors regulating adhesion and biofilm formation by single and dual-species were also studied, particularly the characterization of surface physico-chemical properties of ff through the measurement of contact angles and the evaluation of the adhesion ability by thermodynamic approaches and by *in vitro* assays. The free energy of adhesion due to thermodynamic-based interactions was also assessed as an estimative of the interaction between fungi, bacteria and potential adhesion surface and compared with the results obtained from the *in vitro* adhesion assays. Additionally, single and dual-species biofilm formation in 96-wells polystyrene (PS) microtiter plates was also analysed, in order to characterize the kind of microbial interactions that can occur between ff and the selected bacterium in biofilms.

Furthermore, studies on the effect of sodium hypochlorite (SHC), a disinfectant commonly used in DWDS, on resistance and persistence of single and dual-species biofilms were also performed, in order to understand the role of each microorganism on biofilm susceptibility.

1.3. Thesis organization

This dissertation is composed by 5 chapters. Chapter 1 describes the context, main objectives and motivations for the development of this dissertation and serves as a guideline to the overall work presented in the other chapters.

In chapter 2 a brief review of the literature is provided. The literature review was focused in DWDS problems like biofilm formation and its control. Information on the importance of the study of ff in DWDS is also provided.

Chapter 3 is focused on the study of the adhesion and biofilm formation processes by single and dual-species of the studied microorganisms. In this chapter, information on the surface physico-chemical properties of the microorganisms are presented. Moreover, the adhesion to PS is previewed attending to the microorganisms and PS surface physico-chemical characteristics, based in a thermodynamic approach. Also, the real adhesion to PS is assessed by *in vitro* adhesion assays on microtiter plates as well as the biofilm formation ability.

In chapter 4, both single and dual-species biofilms are subjected to a disinfection treatment using SHC, in order to assess their resistance and persistence to different

concentrations of the disinfectant. The effects of different concentrations (0; 0.1; 0.5; 1; 10 and 100 mg/L) of SHC were analysed according to their ability to inactivate and remove biofilm.

Finally, chapter 5 describes the main conclusions obtained with the work developed in this dissertation and presents some proposals for future research.

Chapter 2

2. Literature review

2.1. Drinking water concerns

Water is fundamental to sustain life and it is a well-known fact that the access to safe DW is crucial to health, a basic human right and a very important issue for an effective policy in health protection. Safe DW is defined by the World Health Organization (WHO) as water that can be used without any risk of immediate or long-term harm (WHO 2011). It is important to notice that the consumption of contaminated DW might cause a variety of health-related problems in all people, especially in the more susceptible such as infants, children, elderly and immunocompromised (WHO 2011).

The supply of clean DW is a major public health milestone (Berry et al. 2006). Thus, there are many regulations with pre-established parameters (including microbiological ones) in order to ensure that the water provided is safe and free of hazards for the human health. However, it is also well-known that many people do not have access to safe DW which is a main concern and a very important reason for all the studies involving this subject (WHO 2011).

According with the state of evolution of each country it is possible to verify that different concepts of DW exist around the world. For developed countries both microbial and chemical contaminations are major concerns, whereas for developing countries, where access to clean water and sanitation are not the rule, the main problems lie with microbial contaminations (Gilbert 2012). Nevertheless, the risk from microbial pathogens remains present for developed countries and the risk of outbreaks of waterborne diseases is not limited to developing countries (Beaudeau et al. 2008; Simões & Simões 2013).

Regarding the origin of chemical contamination, two different sources can be attributed: anthropogenic activities (lead, nitrate, pesticides), and natural occurring inorganic chemicals (arsenic, radon, fluoride) (WHO 2011). Nonetheless, there are also emergent contaminants (antibiotics, human hormones, personal care products and illicit drugs), which are significantly appearing in surface waters (De Gusseme et al. 2011).

The problem with these micropollutants lie with the fact that the conventional processes used for the treatment of DW are not capable of removing them (De Gusseme et al. 2011; Kim et al. 2007). Several chemical contaminants when ingested with DW are responsible for some human health-problems such as vascular disease, dermal lesions, skin and lung cancer, problems in neurodevelopment between others (WHO 2011).

Although chemical contamination is a great issue for developed countries, waterborne diseases are one of the most important health problems associated with water (Beaudeau et al. 2008). Waterborne diseases refer to any illness caused by the utilization of DW contaminated by pathogenic microorganisms like bacteria, protozoa, virus or helminths. Such pathogens are transmitted to the population when untreated or inadequately-treated water is consumed (Simões & Simões 2013). Several diseases are associated with waterborne outbreaks such as cholera, typhoid fever, meningitis, encephalitis, dysentery, hepatitis, legionellosis, pulmonary illness, poliomyelitis, leptospirosis, giardiasis and salmonellosis; being gastroenteritis the most known disease associated with waterborne outbreaks in developed countries (WHO 2011).

It should be noticed that adequate clean water supplies combined with proper sanitation and improved hygiene standards, could significantly reduce the incidence of waterborne diseases (Simões & Simões 2013). Also, DW quality is dependent on the specific composition and activity of microbial populations present during the treatment, storage and distribution phases, which means that microbial contamination is also a problem for DWDS (Farkas et al. 2012). Thus, the creation of more robust and adequate disinfection treatments for DWDS is very important.

2.2. Microbiology of drinking water distribution systems

DWDS can be perceived as environments wherein bacteria, fungi, protozoa, viruses and algae cohabit and interact, making them extremely complex systems (Berry et al. 2006; Hageskal et al. 2009). Therefore, and considering that each microorganism has its own part in this environment, their roles should not be underestimated both as a potential threat to human health or as functional part of an unique ecological niche (Paterson & Lima 2005; Siqueira 2011).

In DWDS about 95% of the total biomass in water is estimated to attach to pipe walls while only 5% is in the water phase (Wingender & Flemming 2004). Furthermore, several problems in DWDS have a microbial source like biofilm growth, nitrification, microbial mediated corrosion and the presence and persistence of pathogens.

Additionally, water microbiology can be affected by several factors, for example: pipe material, nutrient level, water flow, temperature, pH and concentration of disinfectant (Berry et al. 2006).

Usually, in water systems, microorganisms live in association in the form of biofilms, which concedes them increased resistance to adverse factors such as high temperatures, dryness, oligotrophic conditions and biocides when compared with their planktonic form (Davey et al. 2000; Donlan 2002). The presence of biofilm in the pipe walls can lead to lower water quality, not only because there is a biological structure always present, but also because substances from bulk water like chemical contaminants can be imprisoned in the biofilm matrix. Moreover, biofilms can work as a reservoir of pathogens and can stimulate microbial induced corrosion (Szewzyk et al. 2000). Furthermore, the presence of biofilms can alter the taste, odour and the visual appearance of water, which is an indicative of poor DW quality (Gonçalves et al. 2006a).

Concerning the study of biofilms in DW systems, it should be noticed that several errors might occur mainly due to the variations in the scientific methodology used to analyse them (Berry et al. 2006; Hageskal et al. 2009). Another complication in this line of work is the fact that in water networks, the collection of pipes is not easy accessible since it would be necessary their removal, thus *in situ* approaches are normally scarce and pilot systems in laboratory are used instead (Siqueira et al. 2011). These bench top laboratorial devices try to mimic the DWDS behaviour, allowing to test for different conditions and can be fed with tap water or with appropriate medium or enriched water. The development of such devices aims to mimic real DWDS in order to gather results that can be transposed to reality. Hence, the selection and use of an appropriate device is an relevant factor to obtain reproducible and reliable results and should be chosen taking into account the goals of the study (Gomes et al. 2014).

Considering everything that was aforementioned about biofilms in water it is possible to represent them as shown in Figure 1.

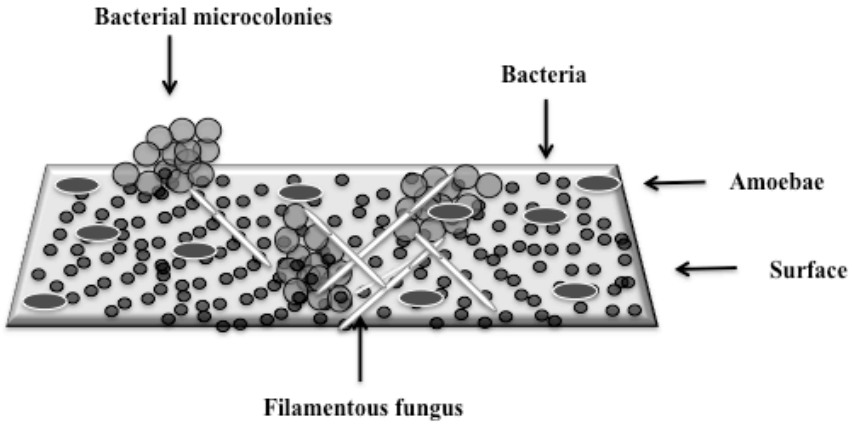

Figure 1- Microbial heterogeneity and architectural structure of water biofilms. Based on Siqueira 2011.

2.2.1. Fungi and filamentous fungi in drinking water

Regarding DW problems it is important to notice that the role played by all fungi in general and ff in particular is still very underestimated and not fully understood (Doggett 2000; Gonçalves et al. 2006b; Hageskal et al. 2009; Paterson & Lima 2005). The main reason for the infrequent discussion on fungi as pathogenic microorganisms in water is related with the fact that the presence of pathogenic bacteria, viruses, and parasites that appear in DW often leads to relatively acute symptoms and disease in humans and so far the consumption of fungal contaminated water has not (Hageskal et al. 2009). However, the presence of fungi in DW might cause organoleptic changes which may lead to poor water quality (Hageskal et al. 2009; Paterson & Lima 2005). Furthermore, it should be noticed that fungi are especially adapted for growth on surfaces as evidenced by their absorptive nutrition mode, their secretion of extracellular enzymes to digest complex molecules, and apical hyphal growth which makes them the perfect candidates for biofilm formation (Jones 1994).

Notwithstanding the fact that no direct association has been made linking fungi with water contamination problems, several studies have been performed displaying that the recovery of fungi in water samples varies between 7.5-89% positive samples (Hageskal et al. 2009). Also, those samples might have a considerable variation in the levels of fungi. It is also important to refer that samples containing fungi have been recovered from many types of water such as raw water, treated water, heavily polluted water, distilled or ultra-pure water and bottled DW (Hageskal et al. 2009).

Considering that microorganisms naturally interact with each other and that

water distribution systems are complex environments full of different microorganisms such as bacteria, protozoa, fungi and virus that compete with each other for nutrition and space, it is expected that bioactive secondary metabolites are produced (Rateb et al. 2013). Mycotoxins are harmful secondary metabolites produced by a wide range of fungi belonging in the main to *Fusarium*, *Aspergillus* and *Penicillium* genera (Bayram & Braus 2012; Reverberi et al. 2010), and as can be seen in Table 1, many water samples present both *Aspergillus* and *Penicillium* fungi. It is of importance to mention that the mycotoxins produced in water are usually extremely diluted and represent a minor concern. However, their concentrations may increase and become an hazard to human health, especially when water is stored in cisterns, reservoirs or even in bottles, for prolonged periods of time (Hageskal et al. 2009).

Fungi in water may be aerosolized into air, and introduced to immunocompromised patients. Furthermore, sensory changes have been associated with the occurrence of fungi in DW systems (Paterson & Lima 2005). Nevertheless, there are still many limitations on fungal water studies especially when it comes to the methodology used. The fact that different methodologies are used for the different studies limits the uniformity between them and makes direct comparisons almost impossible (Hageskal et al. 2009).

In the future, if the microbiological quality of DW is to include fungi, it is most likely that fungal parameters in the water regulations are implemented, including the methods for their detection and quantification. Even though fungi are a difficult group to examine, that should not mean that fungal contamination of DW can be ignored. As it has been mentioned, fungi can affect the water quality in many ways, and therefore the mycobiota of DW should be considered when the microbiological safety and quality of DW is evaluated (Hageskal et al. 2009).

Table 1-Studies performed in different countries where ff were recovered from water supplies

Country, place, year	Type of water	Most frequent fungi isolated	References
Portugal, Braga, 2003-2004	Tap water	*Penicillium* and *Acremonium* spp.	Gonçalves et al. (2006a)
Brazil, Maringá, 2007	Tap and mineral bottled water	Yeasts and filamentous fungi	Yamaguchi et al. (2007)
Norway, 14 networks, 2002-2003	Surface and underground DW	*Penicillium*, *Trichoderma* and *Aspergillus* spp.	Hageskal et al. (2007)
Poland, Warsaw, 2007	Municipal water supply system	*Aspergillus fumigatus* and *Aspergillus niger*	Grabińska-Łoniewska et al. (2007)
Portugal, Lisbon, 2009	Surface, ground and spring water	*Penicillium*, *Aspergillus* and *Cladosporium* spp.	Pereira et al. (2009)
Belgium, Liége, 2010	Tap water from the University Hospital of Liége	*Aspergillus fumigatus* and *Fusarium* spp.	Hayette et al. (2010)

2.2.2. Biofilms

In nature there are significant proportions of microorganisms that persist attached to surfaces in the form of biofilms (Thein et al. 2006). A biofilm is defined as an assemblage of microbial cells that are permanently attached to an abiotic or biotic surface and encircled in a matrix of primarily polysaccharide material (Davey et al. 2000; Donlan 2002; Huq et al. 2008). Extracellular polymers also known as extracellular polymeric substances (EPS) are the key substances keeping biofilm organisms together, gluing them to the surface and providing protection against agents of stress (Simões et al. 2010).

The formation of biofilms may occur on a wide variety of surfaces including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems (Donlan 2002). The solid-liquid interface between a surface and an aqueous medium, such as water, offers an ideal environment for the attachment and growth of microorganisms. Therefore, and in order to understand the attachment of the microorganisms, it is essential to consider the effects and features of the substratum (roughness, hydrophobicity, surface charge, chemical composition), characteristics of the aqueous medium (hydrodynamics, pH, temperature, cations, nutrient availability,

presence of disinfectants), and various properties of the cell surface (hydrophobicity, extracellular appendages, EPS, signalling molecules) (Donlan 2002; Yu et al. 2010). Thus, the cell attachment to a surface and the succeeding biofilm formation can be perceived as a very complex process, with many variables affecting the process. Furthermore, biofilms are considered a stable point in a biological cycle that include roughly four different steps: initiation, maturation, maintenance and dissolution (O'Toole et al. 2000).

In natural habitats as well as in the medical or industrial environments there is a common concern regarding the biofilm mode of growth (Elvers et al. 1998). Consequently, the focus of biofilm research is the understanding of the mechanisms of biofilm formation, structure, detachment and the methods for controlling its growth (Elvers et al. 1998; Siqueira & Lima 2013). However, the great majority of these studies are performed using only pure bacterial cultures, which is not a realistic scenario when compared with what happens in nature (Elvers et al. 1998; Elvers et al. 2001; Gonçalves et al. 2006b). Usually, in natural and industrial environments, multispecies biofilm consortia are found. These multispecies biofilms are normally complex and may even be composed by a large number of species (Elvers et al. 2001; Peters et al. 2010). Although, studies using only bacterial cultures are simpler, it is necessary to better understand the interspecies interactions and how they affect the biofilm to use multispecies consortia (Elvers et al. 2001; Peters et al. 2010). Also, these studies will allow a more realistic scenario.

In order to maintain water quality and preserve human health it becomes essential to control biofilm formation. As stated before, biofilms can act as a reservoir of pathogens and are the origin of several DWDS problems (Szewzyk et al. 2000), hence the importance in its control.

2.2.2.1. Bacterial biofilms in drinking water

As stated previously, biofilms in nature can be composed by several kinds of microorganisms like bacteria, viruses, protozoa, fungi and algae (Momba et al. 2000). Nevertheless, bacteria are generally dominant in biofilms due to their high growth rates, small size, adaptation capacities and the ability to produce EPS (Simões & Simões 2013).

Microbial species are able to come into closer contact with a surface, attach firmly to it, promote cell-cell interactions and grow as a complex structure due to

several mechanisms (Bryers et al. 2004). Biofilm formation comprises a sequence of steps (Bryers et al. 2004), and nowadays, the biofilm formation mechanisms are already described in several reports (Bryers et al. 2004; Chmielewski & Frank 2003; Verstraeten et al. 2008;).

Currently, processes governing biofilm formation that have been identified are the following: 1 - pre-conditioning of the adhesion surface either by macromolecules present in the bulk liquid or intentionally coated on the surface; 2 - Transport of planktonic cells from the bulk liquid to the surface; 3 - Adsorption of cells at the surface; 4 - Desorption of reversibly adsorbed cells; 5 - Irreversible adsorption of bacterial cells at a surface; 6 - Production of cell–cell signalling molecules; 7 - Transport of substrates to and within the biofilm; 8 - Substrate metabolism by the biofilm-bound cells and transport of products out of the biofilm. Accompanied by cell growth, replication, and EPS production; 9 - Biofilm removal by detachment or sloughing (Bryers et al. 2004).

The preconditioning of the adhesion surface is considered the first step in biofilm formation and has to occur before any attachment to the surface by the microorganisms. In this step, a thin layer of organic molecules and ions (preconditioning) will cover the adhesion surface due to physical or chemical adsorption. It should be noticed that the strength of biofilm adhesion is largely dependent on the cohesion of the conditioning film (Bos et al. 1999; Busscher et al. 1995).

After the pre-conditioning of the adhesion surface it is possible to start the adhesion phase, which comprises the steps 2 to 5, previously mentioned. Initially, there is transport of microbial cells to the adhesion surface due to fluid dynamics, gravitational forces and Brownian motion, or by migration through active cell motility. The surface electrostatic charge and hydrophobic interactions affect this first surface-surface interaction and the adhesion process. Cells and the adhesion surface can interact with each other by the establishment of long and short/intermediate distance forces. When these forces are in a favourable equilibrium the end result will be the adhesion of the microorganisms to the surface (Simões 2013).

Following cellular adhesion to surfaces, growth and maturation are the following stages of biofilm formation (previously enumerated as steps 6-9). Firstly, the attached microorganisms start growing and form microcolonies, excrete organic polymers and

initiate the formation of the biofilm matrix (Allison & Sutherland 1987). Transport of nutrients from the external liquid media to the inner layers of biofilm and the transport of excreted metabolites in the opposite direction are important for biofilm maintenance and occur with the increase of thickness. Logically, the maturation phase is the next step. Here occurs the development of complex and organized consortia of microorganisms, which are surrounded by an organic matrix that protects the microorganisms from stress factors. Large amounts of EPS are produced in this stage. Also, in a mature biofilm there is attachment of planktonic bacteria, bacterial detachment into the bulk water, growth and death. Nonetheless, processes are at equilibrium and the number of attached cells per unit surface area is considered constant in time, although fluctuations can occur throughout time (Bryers et al. 2004).

Detachment of cells and other components from the biofilm is the last phase of biofilm formation (step 9). This phenomenon can happen due to different mechanisms such as sloughing (the rapid detachment of large portions of the biofilm), erosion (the continuous release of single cells or small clusters of cells), abrasion (collision of solid particles with the biofilm) and predator grazing (Simões 2013).

2.2.2.2. Filamentous fungi biofilms: can it happen?

As previously mentioned, fungi are excellent candidates for biofilm formation. Nevertheless, the term "biofilm" is rarely used when talking about ff (Harding et al. 2009). Very few reports on filamentous fungal biofilms can be found in the literature, and that is probably due to the fact that ff do not fit completely or precisely within restrictive biofilm definitions which are normally based on bacterial models (Harding et al. 2009). Sometimes to describe the surface-associated growth of ff other terms such as "multicellular masses" and "sub- merged/solid-state fermentation" are used instead of "biofilm" (Gutiérrez-Correa & Villena 2003; Mowat et al. 2009).

Although the reports about ff biofilms are sparse, there are many studies that describe the presence and growth of ff in several environments such as the medical, industrial and environmental (Anaissie et al. 2003; Gutiérrez-Correa & Villena 2003; Mowat et al. 2007). Harding et al. (2009) proposed a set of criteria for ff biofilm formation, and the studies aforementioned meet at least some of the criteria proposed. This criteria is gathered in two groups (i) structural features such as complex aggregated growth, surface-associated growth of cells and secreted extracellular polymeric matrix and (ii) altered gene expression resulting in phenotypic changes that include enhanced

tolerance to antimicrobial compounds or biocides, changes in enzyme or metabolite production and/or secretion and physiological changes (Harding et al. 2009).

Harding et al. (2009) proposed a preliminary model for filamentous fungal biofilm formation, based in some of the already mentioned reports and several others about ff and also drawing from bacterial and yeast models. This preliminary model is divided in six major stages: propagule adsorption, active attachment to a surface, microcolony formation, initial maturation, maturation or reproductive development and dispersal or planktonic phase (Harding et al. 2009). Figure shows the main stages of this preliminary model.

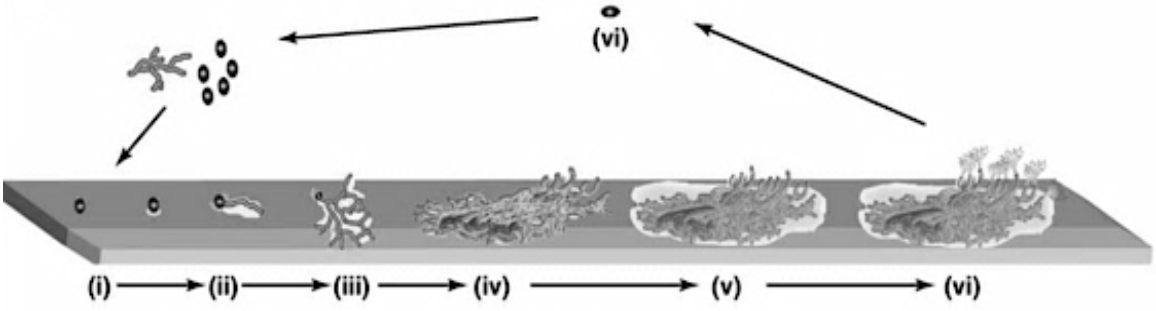

Figure 2- Harding et al. model for ff biofilm formation: (i) adsorption, (ii) active attachment, (iii) microcolony I (germling and/or monolayer), (iv) microcolony II (mycelial development, hyphal layering, hyphal bundling), (v) development of the mature biofilm, and (vi) dispersal or planktonic phase. Adapted from Harding et al. (2009).

The propagule adsorption is comprised by the deposition of spores or other propagules such as hyphal fragments or sporangia. This phase involves mainly physical contact of the ff with a surface. When comparing it with the bacterial models it represents the reversible attachment stage (Harding et al. 2009).

The propagule adsorption is followed by the active attachment to the surface. In this phase usually the ff secrete adhesive substances by germinating spores and active germlings. Also, it is comparable to the fixed attachment phase in bacteria (Harding et al. 2009).

The third phase is the microcolony formation where the initial stages of growth and surface colonization occur. In order to do so the cells produce an extracellular polymeric matrix that allows the growing colony to adhere tenaciously to the substrate (Harding et al. 2009).

Following the microlony formation, the initial maturation happens. This phase encompasses the formation of compacted hyphal networks or mycelia and hypha–

hypha adhesion. Also, includes the layering, the formation of hyphal bundles bonded together by exopolymeric matrix, and the formation of water channels via hydrophobic repulsion between hyphae or hyphal bundles (Harding et al. 2009).

After the initial maturation a stage of maturation occurs. This phase includes the formation of fruiting bodies, sporogenous cells, sclerotia and other survival structures. Aerial growth is often a crucial feature of fungal fruiting and dispersal (Harding et al. 2009).

Dispersal or planktonic phase is the last stage of this preliminary model. This phase is characterized by the dispersal or release of spores or biofilm fragments. The detached cells can act as new propagules to re-initiate the cycle (Harding et al. 2009).

It is important to mention that although most ff do not normally exist as single cells, spores, hyphal fragments and other fungal propagules can be considered functional equivalents to planktonic bacterial cells (Harding et al. 2009).

Studies performed by Beauvais et al. (2007) and Mowat et al. (2009) regarding the ff *Aspergillus fumigatus* characterized compact hyphal balls as biofilms. There was evidence of the presence of an extracellular polymeric matrix, differential gene expression and also a differential sensitivity to antifungal drugs (Beauvais et al. 2007; Mowat et al. 2009;).

2.3. Drinking water distribution systems maintenance

DW is one of the most important resources and when regarding human health it becomes essential to guarantee its quality. Therefore, treatment is a crucial measure in order to guarantee public health security. Also, DW is one of the most closely monitored resources and is subjected to several treatment processes until it can be distributed and consumed (WHO 2011).

Preferably avoiding biofilm formation would be a more logical and suitable option than treating it. Nevertheless, there is currently no known technique that is able to efficaciously prevent or control the formation of unwanted biofilms without causing adverse side effects (Simões et al. 2010). Notwithstanding, there are several measures that can be applied in order to limit biofilm formation. Such measures are the following: minimizing the concentration of organic matter entering the distribution system; ensuring the material from which the pipework and fittings are made so they are both chemically and biologically stable; prevention of water stagnation and sediment

accumulation within the distribution systems; maintenance of a sufficient disinfectant level throughout the distribution system (Simões & Simões 2013; WHO 2011;).

Considering the framework of this thesis only chemical disinfection as a measure to ensure biofilm control and consequently the microbiological DW quality will be discussed.

2.3.1. Disinfection

Water disinfection means the removal, inactivation or killing of microorganisms to guarantee safe water through DWDS from a microbiological point of view. The most used disinfectants are chlorine, chloramines, chlorine dioxide, ozone and UV radiation (Chowdhury 2012). From all the disinfectants chlorine is by far the most widely used in DWDS.

Chlorination is a key step in the biofilm control process. Chlorine, as a strong oxidizing agent, is the most commonly used disinfectant due to its effectiveness, stability, easy of use and low cost (Rand et al. 2007). Chlorination can occur in two distinct points in the treatment process: pre-treatment where a primary disinfection at the beginning of the treatment occurs, and a post-treatment or secondary disinfection aiming to maintain a disinfectant residual in the distribution system. The post-treatment process plays an important role to control the microbial regrowth (Deborde & von Gunten 2008).

Water disinfection is normally achieved by adding disinfectants in excess, particularly chlorine, so that microbial accumulation in pipes and tanks can be controlled. Nevertheless, residual concentrations must be kept below guide-lines to lower the potential to form harmful disinfection by- products (DBP) which are harmful for human health (Rand et al. 2007). Also, high chlorine concentrations can cause organoleptic problems (strong odour and tastes) and lead to the selection of resistant microorganisms (Nieuwenhuijsen et al. 2000).

According to the WHO (2011), 2 to 3 mg/L of chlorine should be added to water in order to achieve satisfactory disinfection and a residual concentration along DWDS. Nonetheless, the maximum amount of chlorine allowed is 5 mg/L. Also, the residual concentration of free chlorine leaving the treatment plant should be less than 1 mg/L and nearer 0.5 mg/L (WHO 2011). However, this residual concentration seems not be enough to stop the growth and development of microbial biofilms (Zhou et al. 2009). It should be noticed that the disinfection with chlorine dioxide and chlorine can reduce the

concentration of planktonic bacteria, but have little to no effect on the concentration of biofilm bacteria (Simões et al. 2010c). This resistance of biofilms to antimicrobials can be due to very low metabolic levels and severely down regulated rates of cell division of the deeply embedded microorganisms. Moreover, biofilms can function as a barrier to antimicrobial agents diffusion (Bridier et al. 2011). It should be noticed that EPS can reduce the disinfectant concentration and their effectiveness since both compounds interact with each other (Bridier et al. 2011).

Chloramines and chlorine dioxide are examples of other oxidant chlorine-based disinfectants used for DW disinfection. Chloramines are less effective than free chlorine and need longer contact times or higher concentrations to achieve the desired disinfection level but create smaller amount of DBP. Yet, this compound is more effective in biofilm penetration than chlorine (Chandy & Angles 2001). The use of chlorine dioxide is not common. Nonetheless, this disinfectant does not produce dangerous DBP (Simões & Simões 2013).

Disinfection can also be performed by non chlorine-based oxidants like ozone. This compound not only efficiently removes microorganisms but also removes odour and taste. Furthermore, ozone produces low amount of DBP. However, this compound is more expensive than chlorine, and its use does not allow for residual disinfection action in DWDS (Simões & Simões 2013).

UV radiation (electromagnetic energy in the range 250-265 nm) can be used as an alternative to chemical disinfection. UV will disinfect by destroying the microorganisms since it will alter their genetic material and render them unable to reproduce. This method is a very effective against all bacteria, viruses and protozoa cysts found in clarified waters. Nonetheless, it presents one major disadvantage which is the fact that UV leaves no residual disinfectant in the water (Liberti et al. 2003). A possible solution for this disadvantage relies on the use of a second disinfectant to generate a residual amount (Simões & Simões 2013).

In the past there was a separation of microbiological research between bacteriologists and mycologists, which led to the study of bacteria and fungi in axenic settings. However, this division is not realistic since in several environments bacteria and fungi coexist and interact (Frey-Klett et al. 2011; Wargo & Hogan 2006). Currently, the studies show that fungi and bacteria frequently form physically and metabolically interdependent consortia that display properties distinct from those of their single components (Tarkka et al. 2009).

2.4. Bacteria and fungi interactions

The bacteria-fungi interactions (BFIs) are of interest for several fields of study such as agriculture, forestry, environmental protection, food processing, biotechnology and medicine (Frey-Klett et al. 2011; Kobayashi & Crouch 2009). The most common reports about BFIs show that normally the bacterial partner exploits resources from the associated fungi through a parasitic or commensalism interaction (Kobayashi & Crouch 2009). Nevertheless, there are examples where the fungi is able to take advantage of bacterial resources in mutualistic interactions (Kobayashi & Crouch 2009).

The physical associations between the complexes formed by bacteria and fungi can range from apparently disordered polymicrobial communities to highly specific symbiotic associations of fungal hyphae and bacterial cells (Frey-Klett et al. 2011). Multispecies biofilms containing both filamentous or non-filamentous fungi and bacteria can be considered as a more intimate level of bacterial-fungal association. This kind of biofilms is different from multispecies communities. Once in the biofilm the microorganisms form structured communities "glued" together by an extracellular matrix of macromolecules produced by the microorganisms, that have physical and physiological properties distinct from those of free-living cells (Donlan & Costerton 2002). BFIs and adhesion are very important early events for the formation of multispecies bacterial-fungal biofilms (Frey-Klett et al. 2011).

The BFIs can be perceived as an equation (Figure 3). In that equation the combination of physical associations and molecular interactions between the two microorganisms can have a variety of different outcomes for each one. These changes may affect the influence of the bacterial-fungal complex on their biotic and abiotic environment (Frey-Klett et al. 2011).

Normally, the microbial communities that exist as a multispecies biofilm present an increased resistance to the treatment with antibiotic and antifungals. This resistance might be due not only to a more complex polymeric matrix composition, but also to the fact that has been proposed that antimicrobial resistance profiles change in multispecies infections (Wargo & Hogan 2006). Therefore, since the biofilms in DWDS are a complex environment, where the bacteria-fungal biofilms are present, the BFIs could play a significant role on biofilm formation and control in these systems. So, their knowledge is essential to improve DW quality.

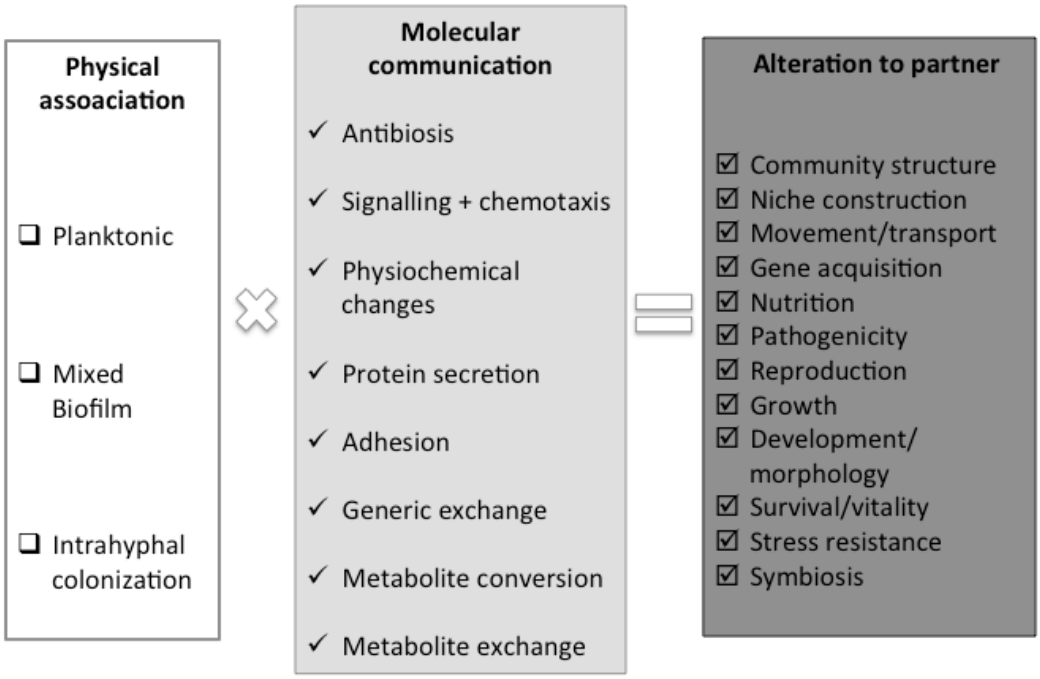

Figure 3- BFIs equation. Adapted from Frey-Klett et al. (2011).

Chapter 3

3. Single- and dual-species adhesion and biofilm formation by *Acinetobacter calcoaceticus*, *Penicillium expansum* and *Penicillium brevicompactum*

3.1. Introduction

Microbial adhesion is the initial step in biofilm formation. Adhesion intensifies contamination of DW, reduces the aesthetic quality of potable water, increases the corrosion rate of pipes and decreases microbiological safety since it can intensify the survival of pathogens (Simões et al. 2010a).

As mentioned previously, the development of a biofilm is believed to occur in a sequential process that includes transport of microorganisms to surfaces, initial reversible/irreversible adhesion, cell-cell communication, formation of microcolonies, EPS production and biofilm maturation (Bryers et al. 2004; Doyle 2000). Consequently, cell adhesion to the surface is one of the main steps in biofilm formation.

Bacteria-surface adhesion can be explain by several approaches like the Derjaguin-Landai-Verwey-Overbeek (DLVO) theory, thermodynamic approaches and the extended DLVO (XDLVO) theory (Simões et al. 2010a). Nevertheless, it should be noticed that these approaches do not consider some bacterial characteristics, such as the production of extracellular substances (EPS and lipopolysaccharides – LPS), the presence of appendages (fimbriae, pili, flagellum) and the ability of microorganisms to regulate differently their gene expression when facing different environmental conditions (An & Friedman 1998). Furthermore, cell adhesion can be influenced by several factors, like the physico-chemical characteristics of microorganisms (hydrophobicity, surface charge), the material surfaces properties (chemical composition, surface charge, hydrophobicity, roughness and texture) and by the environmental factors (temperature, pH, time of exposure, cell concentration, chemical treatment or the presence of antimicrobials and fluid flow conditions) (An & Friedman 1998).

For microbial adhesion, a two-phase process has been described (Pavithra & Doble 2008). Briefly, initial adhesion between microorganisms and non-living surfaces

is usually mediated by physical forces, such as brownian motion, van der Waals attraction forces, hydrogen bonding, acid-base, electrostatic interactions, gravitational forces, hydrophobic interactions and biospecific interactions (Simões et al. 2010a). Magnitudes of these forces are affected by the distance of the microorganisms from the surface (long-range and short-range forces) and by ionic strength. Later the molecular reactions between microbial surface structures and substratum surfaces become predominant. This implies a firmer adhesion of microorganisms to the surface (Simões et al. 2010a). It should be noticed that these studies were performed using bacteria and, little is known both about the adhesion process of ff as about their physical-chemical properties.

Regarding ff and adhesion it is known that these microorganisms produce hydrophobins. The hydrophobins form an amphipathic membrane whose hydrophobic side is exposed to the exterior and the hydrophilic surface is connected to the cell wall polysaccharides providing water repellent properties. Hydrophobicity seems to mediate attachment of hyphae to hydrophobic surfaces and can also function in cases of symbiosis between fungi and plants (ectomycorrhizae) or algae and/or cyanobacteria (lichens) (Linder et al. 2005).

In water-solid interface, hydrophobic interactions are of crucial importance for the achievement of firm adhesion of microorganisms (Donlan & Costerton, 2002). Several bacterial and fungal pathogens rely on hydrophobic interactions for an effective colonization of the host even though this effect has been considered nonspecific (Doyle 2000). Furthermore, fungal-bacterial biofilms can be mediated by hydrophobic and electrostatic interactions where the fungal cells provide a surface for bacteria to adhere on (Morales et al. 2010).

Chau et al. (2009; 2010) and Siqueira et al. (2012) published recently some studies about hydrophobicity in ff. Nonetheless, these are one of the only reports about the topic mainly due to the delicate and multifaceted nature of fungal growth (Chau et al. 2009).

As previously mentioned, in DWDS are environments wherein bacteria, fungi, protozoa, viruses and algae cohabit and interact (Berry et al. 2006; Hageskal et al. 2009). Usually these microorganisms live in association in the form of biofilms, which concedes them increased resistance to adverse factors (Davey et al. 2000; Donlan 2002). The presence of biofilms can alter the taste, odour and the visual appearance of water

which is not desirable when considering a good quality DW (Gonçalves et al. 2006b).

In this chapter the adhesion process for *A. calcoaceticus*, *P. expansum* and *P. brevicompactum* is presented. The sessile drop contact angle method was used to quantify the surface hydrophobicity and free energy of adhesion of both *A. calcoaceticus* and fungal spores. Fungal hydrophobicity was also assessed qualitatively using mycelial mats on solid culture. Furthermore, the adhesion and the biofilm formation of single and dual-species biofilms was performed using 96 wells PS microtiter plates. The biofilms were analysed by biomass quantification assays (using CV) and by metabolic activity assays (using resazurin). Both studies are of crucial importance considering the lack of information regarding adhesion and the biofilm formation process for single and dual-species consortia involving ff.

3.2. Materials and methods

3.2.1. Microorganisms and culture conditions

The bacteria used in this study, *A. calcoaceticus*, was isolated from a DWDS and identified by 16S rRNA gene sequencing as described by Simões et al. (2007). Bacterial cells were grown overnight in batch culture using R2A broth (0.5 g/L yeast extract; 0.5 g/L proteose peptone; 0.5 g/L casein hydrolysate; 0.5 g/L glucose; 0.5 g/L starch soluble; 0.3 g/L sodium pyruvate, 0.3 g/L di-potassium hydrogen phosphate; 0.05 g/L magnesium sulphate 7 H_2O) at 25 °C and under agitation (150 rpm) in an orbital incubator (New Brunswick Scientific, I26, USA). *A. calcoaceticus* was harvested by centrifugation (Eppendorf centrifuge 5810R) during 12 minutes at 3777 g, followed by two washes in phosphate-buffered saline (0.2 M PBS, pH 7) and resuspended in the same buffer. Afterwards, the optical density (O.D.) (610 nm) was adjusted to 0.4. The cells were then used for contact angle measurements.

P. brevicompactum and *P. expansum* were supplied by Micoteca da Universidade do Minho fungal culture collection (MUM, Braga, Portugal), and were selected based on their high occurrence in the tap water of the north of Portugal (Gonçalves et al. 2006a). Stock solutions of *P. brevicompactum* and *P. expansum* were obtained from a 7-d pure culture in malt extract agar (MEA: 20 g/L malt extract; 5 g/L mycological peptone; 20 g/L agar) at 25 °C by adding 2 mL of sodium chloride (0.85%)-Tween 80 (0.05%) solution to the plate. The spore suspension collected was then inoculated in a sterile Erlenmeyer flask with solid MEA and grown for 7 d at 25ºC.

After that time, approximately 25 mL of sodium chloride (0.85%)-Tween 80 (0.05%) solution was added to the flask and subsequently a vigorous agitation was performed (with the aid of a magnetic stir bar). A suspension (containing spores and mycelia) was collected. The spores were gathered by filtering the initial suspension in a system with glass wool. Spore quantification was performed using a Neubauer counter chamber and the final solution was cryopreserved (-80 °C) in small aliquots with 10% glycerol.

3.2.2. Formation of mycelial mats on solid culture

For the formation of mycelial mats on solid culture the procedure proposed by Chau et al. (2009) was mostly followed. Firstly, sterile microscope slides were used and transferred aseptically to sterilized Petri dishes. Approximately 1 mL of the MEA was spread uniformly on the slide using a micropipette. The slide medium was then allowed to harden. Under aseptic conditions, a small scraping of fungi from an actively growing culture (7 d old) was inoculated on the centre of the slide medium and incubated at room temperature for 7 d.

3.2.3. Formation of spore mats

For the formation of spore mats a novel technique was developed (based on existent technique for bacterial cells) consisting in the formation of a uniform mat of spores on membrane filtration by filtering 10 mL of a spore suspension (1×10^5 spores/ml in saline solution) as described in section 3.2.4. The spore suspension was prepared under sterile conditions using the spore stock solutions obtained as explain in section 3.2.1.

3.2.4. Surface hydrophobicity and free energy of adhesion

The surface tension of the bacterium and of fungal spores' surface were determined using the sessile drop contact angle method. Bacterial and spore lawns were prepared for contact angle measurements according to Simões et al. (2007a). Briefly, the bacterial cells and fungal spore suspensions previously prepared as described in Section 3.2.1 and 3.2.3 were filtered (50 mL for bacterial cells and 10 mL for fungal spore suspension) through a membrane (0.45 μm, Whatman) in order to achieve a uniform layer of cells. Additionally, the hydrophobicity of fungi was also determined on mycelial mats on solid culture using the sessile drop contact angle method. In this case, the contact angles were measure at the edge, middle and centre points of the mycelial mats which represent the ages of the fungal colonies (from the youngest to the oldest

zones, respectively). Contact angle measurement > 90° were interpreted as hydrophobic property of the surface (Chau et al. 2009). The measurements were performed at room temperature (23 ± 3 °C) using three different liquids, water, formamide and α-bromonaphtalene (Sigma, Portugal), with exception of the mycelial mats on solid culture where was only used water since readings with formamide and α-bromonaphtalene were not possible. The contact angles were determined using a model OCA 15 Plus (DataPhysics, Germany) video based optical contact angle measure instrument, allowing image acquisition and data analysis. The reference liquids surface tension components were obtained from literature (Janczuk et al. 1993).

Hydrophobicity was assessed following the van Oss et al. approach (1987, 1988 and 1989). According to this approach, the degree of hydrophobicity of a given surface (s) is expressed as the free energy of interaction between two entities of that surface, when immersed in water (w) - ΔG_{iwi} (mJ/m^2). When $\Delta G_{iwi}^{TOT} < 0$, the interaction between the two entities is stronger than the interaction of each entity with water and the material is considered hydrophobic. If $\Delta G_{iwi}^{TOT} > 0$, the material is hydrophilic. ΔG_{iwi} can be calculated using the surface tension components of the interacting entities, according to to Equation 1:

$$\Delta G_{iwi}^{TOT} = -2\left(\sqrt{\gamma_i^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_i^+\gamma_w^-} + \sqrt{\gamma_i^-\gamma_w^+} - \sqrt{\gamma_i^+\gamma_i^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad (1)$$

where γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy, and γ^+ and γ^- are the electron acceptor and electron donor parameters of the Lewis acid-base component (γ^{AB}), respectively, being $\gamma^{AB} = 2\times\sqrt{\gamma^+\gamma^-}$.

The surface tension components of a surface (microorganisms or substratum) were obtained by measuring the contact angle of the referred three pure liquids with the surface tension components (which are well-known) and by simultaneous resolution of three equations in the form of Equation 2.

$$(1 + cos\theta)\gamma_i^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_i^{LW}} + \sqrt{\gamma_s^+\gamma_i^-} + \sqrt{\gamma_s^-\gamma_i^+}\right) \quad (2)$$

where θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$.

The interaction between substrate i and v that are immersed or dissolved in water is represented by the total interaction energy (ΔG_{iwv}^{TOT}) and can be expressed by Equation 3.

$$\Delta G_{iwv}^{TOT} = \gamma_{iv}^{Lw} - \gamma_{iw}^{Lw} - \gamma_{vw}^{Lw} + 2\left[\sqrt{\gamma_w^+}\left(\sqrt{\gamma_i^-} + \sqrt{\gamma_v^-} - \sqrt{\gamma_w^-}\right) + \sqrt{\gamma_w^-}\left(\sqrt{\gamma_i^+} + \sqrt{\gamma_v^+} - \sqrt{\gamma_w^+}\right) - \sqrt{\sqrt{\gamma_i^+\gamma_v^-}} - \sqrt{\sqrt{\gamma_i^-\gamma_v^+}}\right] \quad (3)$$

From a thermodynamics point of view, if $\Delta G_{iwv}^{TOT} < 0\ mJ/m^2$ adhesion is favourable, but if $\Delta G_{iwv}^{TOT} > 0\ mJ/m^2$ then adhesion is not expected to occur.

3.2.5. Adhesion and biofilm formation

Both adhesion and biofilm formation assays (single and dual-species) were performed using sterile 96-wells PS microtiter plates (Orange Scientific, USA).

For single adhesion and biofilm formation with *A. calcoaceticus* the protocol described by Simões et al. (2010a) was followed. Briefly, the inoculum was prepared using an overnight culture (as described in section 3.2.1) and adjusting its OD_{620nm} to 0.04 ± 0.02 with fresh R2A broth. Afterwards, 200 µL of cell suspension were added to at least 16 wells of a sterile 96-wells PS microtiter plate. To promote adhesion and biofilm formation, the plates were incubated aerobically on a shaker at 150 or 25 rpm, at 25 °C, for 2 (adhesion), 24 and 48 h (biofilm formation) in accordance with (Simões et al. 2010a). Each 24 h the growth medium was carefully discarded and replaced by fresh one. Regarding single fungal adhesion and biofilm formation a similar protocol was used with some modifications. *P. expansum* and *P. brevicompactum* spore suspensions with 1×10^5 spores/ml in R2A broth were used. In this case, the adhesion time was 4 h. The medium was not changed by fresh one after 24 h. For dual-species (bacteria-fungi) adhesion and biofilm formation assays, 100 µL of suspension of each species of interest was added to the well (making-up to 200 µL). Afterwards the same protocol as described before was followed and adhesion times of 2 and 4 h were tested. Negative controls for all assays were obtained by incubating the wells only with R2A broth without adding any bacterial cells/fungal spores.

For all assays, after the desired incubation period, the content of each well was removed and the wells were washed at least two times with 250 µL of sterile distilled water to remove reversibly adherent microorganisms. Then, the remaining attached microorganisms were analysed in terms of biomass adhered on the inner walls of the wells, and in terms of their metabolic activity.

3.2.6. Metabolic activity assessment using alamar blue/resazurin

The main component of alamar blue is resazurin, which is a blue redox indicator that can be reduced to pink by viable microorganisms in the biofilm. Therefore, growth can maintain a reduced environment and the extent of conversion from blue to pink reflects the cell viability (Extremina et al. 2011).

The metabolic activity was assessed using resazurin. In order to do so, a commercially available resazurin solution (Sigma) was used. Stock solutions (400 μM in water) were stored at -20 °C. For this method, 190 μL of sterile R2A broth were added to all wells followed by the addition of 10 μL of resazurin stock solution. Microtiter plates were incubated at 25 ºC, in the dark. Fluorescence (λ_{ex}: 530 nm and λ_{em}: 590 nm) was measured 1 and 18 h after incubation (for bacteria and fungi, respectively) using a microtiter plate reader (SpectraMax M2E, Molecular Devices). For the trials using dual-species the incubation period was of 1 h, since there are evidences that the incubation period for these kind of assays, where fungi are mixed with bacteria are significantly lower than for trials using only fungi (unpublished data). The specific metabolic activity was presented as the ratio between the biofilm metabolic activity per unit of biofilm mass.

3.2.7. Biomass quantification using crystal violet

CV is a basic dye capable of binding to negatively charged surface molecules and polysaccharides in the extracellular matrix of both live and dead cells. Therefore, it can be used to quantify the matrix of both live and dead cells (Extremina et al. 2011).

Firstly, 250 μL of methanol (VWR, Merck) were added for 15 min to promote cell/spore fixation to the wells. Afterwards, supernatants were removed and the plates were air-dried. The fixed microorganisms were stained for 5 min with 200 μL/well of CV solution (Gram colour-staining set for microscopy, Merck) and washed abundantly with water. Afterwards, the plates were left to dry and, finally, the dye bound to the adherent cells was resolubilized by 200 μL of acetic acid 33% (v/v) (VWR, Portugal). The O.D. of the obtained solutions was measured at 570 nm using a microtiter plates reader (Bioteck Synergy HT) and biofilm mass was presented as $O.D._{570\ nm}$ values.

3.2.8. Adherent/biofilm microorganisms classification

Bacteria and fungi were classified using the scheme of Stepanovic et al. (2000)

as described below:

- non-adherent/non-biofilm producer (0): O.D. ≤ $O.D._c$
- weakly adherent/weak biofilm producer (+): $O.D._c$< O.D. ≤ 2 × $O.D._c$;
- moderately adherent/moderate biofilm producer (++): 2 × $O.D._c$ < O.D. ≤ 4 × $O.D._c$;
- strongly adherent/strong biofilm producer (+++): 4 × $O.D._c$ < $O.D._.$.

This classification was based upon of cut-off of the O.D. ($O.D._c$) value defined as three standard deviation values above the mean of O.D. of the negative control (Simões et al., 2010a).

3.2.9. Statistical analysis

The data was analysed using One-Way Anova. Significance level for the difference between data was set at $p < 0.05$. The data were analysed using the statistical program Prism 6. It should be noticed that for each condition tested three replicates were performed.

3.3. Results and discussion

3.3.1. Surface physico-chemical properties and free energy of adhesion

Microbial adhesion can be influenced by the surface physico-chemical properties of microorganism and substratum involved on the adhesion process. The surface hydrophobicity for both bacterium and fungal spores was determined using the approach of van Oss (1997), which allows the assessment of the absolute degree of hydrophobicity of any surface in comparison with their interaction with water. This approach is broadly applied to assess the bacterial hydrophobicity. However, no studies of its application to ff cells were found in the literature. This can be explained by the heterogeneous growth of ff. Nevertheless, taking into consideration the fact that in this study the spores are the initial structures for adhesion and subsequent biofilm formation, it becomes very important to gather information on their physico-chemical characteristics, namely their hydrophobicity. It should be noticed that hydrophobic interactions are of crucial importance in the firm adhesion of diverse microorganisms to water-solid interfaces (Donlan & Costerton 2002). Table 2 shows the surface tension parameters, hydrophobicity and free energy of adhesion for *A. calcoaceticus*,

P. expansum and *P. brevicompactum*. The values showed for the surface tension parameters of PS were obtained from Simões et al. (2010a). Additionally the values obtained for contact angles (in degrees) measured with water, formamide and α-bromonaphtalene for all assays can be found in Appendix A.

Table 2- Values of surface tension parameters (γ^{LW}- Lifshitz- van der Waals component; γ_i^+- electron acceptor parameter; γ_i^-- electron donor parameter), hydrophobicity of microorganisms (i) when immersed in water (w) (ΔG_{iwi}^{TOT}) and free energy of adhesion (ΔG_{1w2}^{TOT}) between the microorganisms (1) and PS (2) in water (w) The results presented are means ± SDs of three independent experiments

	Surface tension parameters (mJ/m^2)			**Hydrophobicity (mJ/m^2)**	**Free energy of adhesion (mJ/m^2)**
	γ_i^{LW}	γ_i^+	γ_i^-	ΔG_{iwi}^{TOT}	ΔG_{1w2}^{TOT}
Bacteria					
A. calcoaceticus	25.57	5.19	44.20	17.42	4.40
Fungal spores					
P. expansum	41.79	0.00	37.41	15.10	-14.10
P. brevicompactum	41.66	0.22	23.05	-10.92	-25.60
Surface					
PS	39.00	0.00	9.90	-44.00	

Based on the van Oss (1997) approach the surface of *A. calcoaceticus* is hydrophilic ($\Delta G_{iwi}^{TOT} > 0$ mJ/m^2). These results are in accordance with these present by Simões et al. (2010a) which also showed *A. calcoaceticus* surface as being hydrophilic. Furthermore, *A. calcoaceticus* also appears to be an electron donor (γ_i^-), presenting also the ability of accepting electrons (γ_i^+).

Regarding the results obtained with the spores of *P. brevicompactum* and *P. expansum* and according with the van Oss (1997) approach, it is possible to characterize *P. expansum* spores as hydrophilic ($\Delta G_{iwi}^{TOT} > 0$ mJ/m^2) and *P. brevicompactum* spores as hydrophobic ($\Delta G_{iwi}^{TOT} < 0$ mJ/m^2). The characterization obtained with this technique for *P. expansum* spores appears to be vague since there are reports in literature were, not only spores of *P. oxalicum* but also spores of *P. expansum* were characterized as hydrophobic (Pascual et al. 2000; Amiri et al. 2005). However, the characterization obtained for *P. brevicompactum* is in accordance with the available literature (Pascual et al. 2000; Amiri et al. 2005). Additionally, both fungi appear to be electron donors (γ_i^-), and *P. brevicompactum* presents the ability of accepting electrons (γ_i^+) while *P. expansum* does not present such capability.

The ability of the microorganisms to adhere to a PS surface can be predicted by the free energy of interaction between the microorganism and the surface when immersed in water, calculated according to the thermodynamic approach. The results obtained show that *A. calcoaceticus* has positive values of free energy of adhesion, which means that adhesion is not thermodynamically feasible (Simões et al. 2010a). These results are in concordance with what has already been described in previous works for the adhesion of this specific bacteria to PS surface (Simões et al. 2010a). Additionally, both spores presented negative values of free energy of adhesion, which indicates that adhesion is thermodynamically possible.

As previously mentioned, surface hydrophobicity of the ff was also characterized by measuring contact angles of mycelial mats on solid culture by following the protocol developed by Chau et al. (2009). The results obtained are presented in Table 3.

Table 3- Contact angles with water (θ_w) for mycelial mats on solid culture (MEA) grown for 7 d. The results presented are means ± SDs of three independent experiments

Fungus	**Colony zone**	**Contact angle (º)** (θ_w)
P. expansum	Centre	ND
	Middle	101.70 ± 7.24 °
	Edge	99.67 ± 5.34 °
P. brevicompactum	Centre	ND
	Middle	ND
	Edge	108.01 ± 5.78 °

ND – Not determined.

For both fungi it was not possible to determine contact angle values in the centre (older zones) of the colonies since the water drop could not be released from the dispenser tip (data shown in Appendix section B). A possible explanation for this phenomenon relies on the fact that a high number of spores can be found in the oldest zones of the colonies, which are responsible for the very high hydrophobicity observed. Also, as reported in the literature, the water drop was covered with spores after contact with the colony surface (Siqueira & Lima 2012). Furthermore, and for the same reason, for colonies of *P. brevicompactum* it was also not possible to perform the contact angles measurements in the middle region.

The values obtained for the contact angles in both fungi are above 90º and

therefore the surfaces can be characterized as hydrophobic. These results are in agreement with what has been reported for these fungi and solid medium (Siqueira & Lima 2012). Furthermore, high hydrophobicity was also reported in 10 d cultures of *P. auranteogriseum* in potato dextrose agar (Chau et al. 2009).

In order to assess the hydrophobicity of mycelial mats on solid culture in a poor medium commonly used in studies with DW-isolated microorganisms, R2A was also used to growth the fungi. However, the measurements were not possible due to the irregular growth that both fungi exhibited, as can be observed in appendix section C to *P. expansum*. Such behaviour can be explained by the fact that the levels of nutrients can interfere with the ff colony development (Smits et al. 2003).

3.3.2. Adhesion and biofilm formation

3.3.2.1. Biomass quantification

Biomass quantification/productivity was assessed using CV dye in order to study the adhesion and biofilm formation. The values obtained for productivity (in terms of $O.D._{570\ nm}$) for all the microorganisms and microbial consortia tested (according to the incubation period) are shown in Figure **4**a for 150 rpm and in Figure **4**b for 25 rpm.

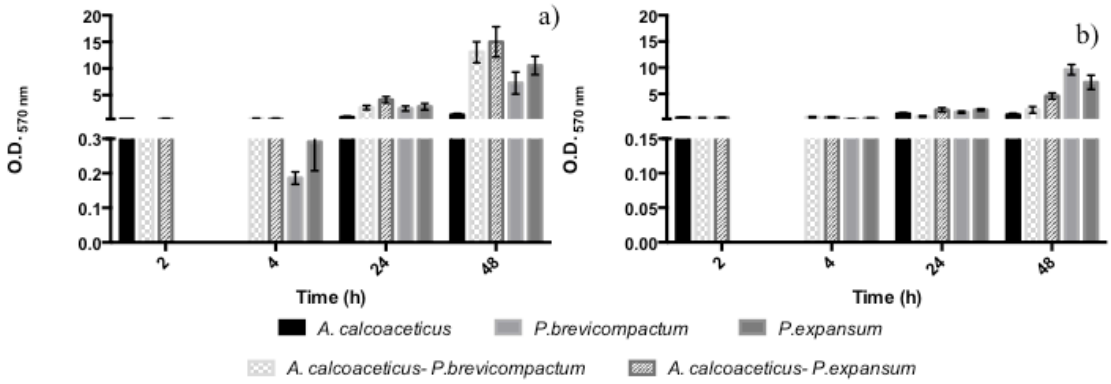

Figure 4 – Biomass productivity values in terms of $OD_{570\ nm}$ as a measure of single and dual-species adherent/biofilm mass overtime for growth conditions at 150 rpm (a) and 25 rpm (b).

The classification scheme proposed by Stepanović et al. (2000) was used to more easily compare the biomass quantification for both adhesion and biofilm formation between the different species and microbial combinations tested. The results obtained for adherent cells and biofilms formed at 150 rpm using such classification are presented in Table 4.

Table 4- Adhesion and biofilm formation ability of the microorganisms grown at 150 rpm. For *A. calcoaceticus* values for the adhesion after 4 h were not collect and the same occurred for *P. brevicompactum* and *P. expansum* for 2 h of adhesion [a]

Microorganisms	Adhesion at sampling time (h)		Biofilm formation at sampling time (h)	
	2	4	24	48
A. calcoaceticus	+		++	+++
P. brevicompactum		0	+++	+++
P. expansum		+	+++	+++
A. calcoaceticus- P. brevicompactum	+	+	+++	+++
A. calcoaceticus- P.expansum	+	+	+++	+++

a- According to classification proposed by Stepanović et al. (2000): (0) non-adherent/non-biofilm producer; (+) weakly adherent/weak biofilm producer; (++) moderately adherent/moderate biofilm producer; (+++) strongly adherent/strong biofilm producer.

For 150 rpm and comparing the results obtained for adhesion and biofilm formation for both fungi, it is possible to verify that after a 4 h period there are no significant changes (p >0.05) between the $O.D._{570}$ values obtained. According to the classification proposed by Stepanović et al (2000) *P. expansum* can be characterize as weakly adherent and *P. brevicompactum* as non-adherent when 150 rpm are used. One explanation is that not many structures have adhered to the well surface. Siqueira & Lima (2013) also described that for *Penicillium* sp. no adhesion was found before a 8 h period. Furthermore, and for 24 h both fungi had similar biomass (p > 0.05) and were both strong biofilm producers. These results are in accordance to the previous report of Siqueira & Lima (2013). These authors found the existence of *Penicillium* sp. monolayers 24 h after incubation. Nonetheless, even though that, according to the classification proposed by Stepanović et al (2000), both fungi were strong biofilm producers. For the 48 h incubation period, it is possible to verify that *P. expansum* presented higher values of biofilm mass when compared with *P. brevicompactum* (p <0.05).

Concerning the results obtained for *A. calcoaceticus* it is possible to verify that for a 2 h period the bacterium is weakly adherent. Regarding the biofilm formation it is possible to verify that for 24 h incubation, the bacterium is a moderate biofilm producer and for 48 h it is a strong biofilm producer.

Analysing the results obtained for dual-species adhesion and biofilm formation of *P. brevicompactum-A. calcoaceticus* with those obtained for single species of *A. calcoaceticus*, it is possible to verify that no significant difference can be perceived ($p > 0.05$) for the $O.D._{570}$ values obtained after 2 h. This behaviour can be due to the bacterial adhesion process. In fact Siqueira & Lima (2013) verified that *Penicillium* sp. adhesion occurred in an 8 h period. *A. calcoaceticus* adhesion to PS after 2 h can be found in the work of Simões and co-workers (Simões et al. 2010a). For both 24 and 48 h incubation periods, biofilm mass was significantly higher for the dual-species biofilms ($p < 0.05$) when compared with *A. calcoaceticus* single biofilms. As for the results obtained for dual-species adhesion and biofilm formation of *P. brevicompactum* and *A. calcoaceticus* with those obtained for single species of *P. brevicompactum*, it is possible to verify that no significant differences can be observed ($p > 0.05$) for the $O.D._{570}$ values obtained for 4 and 24 h incubation periods between each consortium. These results might indicate that the presence of *A. calcoaceticus* does not alter, for the initial periods, the productivity of the dual-species consortium. For 48 h, the dual-species biofilms presented higher values of biomass when compared with *P. brevicompactum* single biofilms. Conversely to what was previously supposed, after 48 h it is possible that the presence of *A. calcoaceticus* influenced positively biofilm productivity.

Considering the results obtained for dual-species adhesion and biofilm formation of *P. expansum-A. calcoaceticus* and comparing with those obtained for *A. calcoaceticus*, it is possible to verify that for 2 h incubation there is no significant difference between the values of $O.D._{570}$ ($p > 0.05$). However, for the 24 and 48 h growth periods considerably higher values were obtained ($p < 0.05$). Therefore, and bearing in mind the fact that the same behaviour occurred with the dual-species biofilms with *P. brevicompactum,* the same conclusions drawn previously can also be applied for this situation. Regarding the comparison between the aforementioned dual-species and single species *P. expansum,* it is possible to verify that for 4 h growth there is no significant difference between both ($p > 0.05$), as occurred with dual-species consortium with *P. brevicompactum* and its single species biofilm. Nonetheless, for the 24 and 48 h incubation periods considerably higher values of $O.D._{570}$ are found for the dual-species biofilms ($p < 0.05$). Thus, it is possible to assume that *A. calcoaceticus* had a higher influence in the productivity of dual-species biofilms of *P. expansum-A. calcoaceticus* than for the dual-species biofilm with *P. brevicompactum*.

Comparing both dual-species sessile cells, 2 and 4 h old, it is possible to find similar values ($p > 0.05$) for both cases. This fact might mean that the initial steps for adhesion are similar for both consortia. However, 24 and 48 h latter higher values of biofilm mass existed for dual-species biofilms of *P. expansum- A. calcoaceticus*.

Regarding the classification scheme proposed by Stepanović et al. (2000) it is possible to verify that after 48 h all consortia are strong biofilm producers.

The adhesion and biofilm formation assays were also performed at 25 rpm. The results obtained using the classification scheme proposed by Stepanović et al. (2000) for this set of trials are shown in Table 5.

Table 5- Adhesion and biofilm formation ability of the microorganisms grown at 25 rpm. For *A. calcoaceticus* values for the adhesion after 4 h incubation were not collected and the same occurred for *P. brevicompactum* and *P.expansum* for 2 h of adhesion [a]

Microorganisms	Adhesion at sampling time (h)		Biofilm formation at sampling time (h)	
	2	4	24	48
A. calcoaceticus	+		+++	++
P. brevicompactum		0	+++	+++
P. expansum		+	+++	+++
A. calcoaceticus- P. brevicompactum	+	+	+++	+++
A. calcoaceticus- P. expansum	+	+	++	+++

a- According to classification proposed by Stepanović *et al* (2000): (0) non-adherent/non-biofilm producer; (+) weakly adherent/weak biofilm producer; (++) moderately adherent/moderate biofilm producer; (+++) strongly adherent/strong biofilm producer.

For 25 rpm, single species adhesion of *P. brevicompactum* and *P. expansum* presented no significant difference in biomass quantification for 4 h of incubation, as occurred for 150 rpm. According with the classification scheme proposed by Stepanović et al. (2000), it is possible to assess that *P. expansum* is weakly adherent and *P. brevicompactum* can be characterized as non-adherent. Once again this can be due to the fact that no significant adhesion is expected to occur before 8 h of incubation for *Penicillium* sp. (Siqueira & Lima 2013). Additionally, for the 24 h incubation period both fungi presented similar values of $O.D._{570}$ ($p > 0.05$) as occurred for 150 rpm. Conversely, after 48 h, it is possible to attest that *P. brevicompactum* presented higher values for biofilm mass quantification when compared with *P. expansum* ($p < 0.05$). For

both incubation periods and according with the classification proposed by Stepanović et al. (2000) both fungi were strong biofilm producers. Therefore, one can conclude that *P. brevicompactum* seems more fit to grow under low agitation speeds while *P. expansum* thrives under higher agitation speeds.

For the results obtained with *A. calcoaceticus* it is possible to verify that after a 2 h period the bacterium is weakly adherent. However, regarding the biofilm formation it is possible to verify that for 24 h incubation the bacterium is a strong biofilm producer and for 48 h there is a decrease in biofilm production becoming this bacterium a moderate producer.

Comparing the values obtained for $O.D._{570}$ of the dual-species adhesion and biofilm formation of *P. brevicompactum-A. calcoaceticus* and *A. calcoaceticus*, it is possible to verify that for all incubation periods tested, the dual-species and single species association behaved similarly to what happened at 150 rpm for the same consortia (except for a 24 h incubation period), and therefore the same conclusions can be established for both 4 and 48 h incubation periods. Significantly higher values of biofilm mass were found for 24 h old *A. calcoaceticus* single biofilms ($p < 0.05$), which might indicate that for 25 rpm, the association between fungi and bacteria at initial periods is less productive. Concerning the results obtained for the adhesion and biofilm formation of the dual-species aforementioned and comparing them with the ones obtained for single species *P. brevicompactum*, significantly higher values of biomass were found for single species *P. brevicompactum* for 24 and 48 h aged biofilms ($p < 0.05$). These results seem to indicate that under low agitation speeds the presence of the bacterium in the biofilm might influence negatively its productivity. Nonetheless, and after a 4 h adhesion period, similar values were found for both consortia ($p > 0.05$).

Performing a similar analysis for the adhesion and biofilm formation of dual-species *P. expansum-A. calcoaceticus* and *A. calcoaceticus* single species, no significant differences in biomass quantification can be perceived after a 2 h adhesion period ($p > 0.05$). For both 24 and 48 h growth periods higher values for biomass quantification were found for dual-species biofilms ($p < 0.05$). Therefore, and considering that a similar behaviour can be found for the same microorganism under 150 rpm the same conclusions can be drawn in this case. Concerning the values obtained for dual-species previously mentioned and single species *P. expansum* no significantly differences were observed when they were 4 and 24 h old. However, for 48

h old biofilms, higher values of mass were attained for *P. expansum,* which can once again indicate that the presence of the bacterium in biofilm, under low agitation speeds, can negatively affect biofilm productivity.

For both dual-species sessile cells, it is possible to conclude that similar values ($p > 0.05$) were obtained when they were 2 and 4 h old. This fact might mean that the initial steps for adhesion are similar for both dual-species consortia. After 24 and 48 h higher values for biomass quantification exist for dual-species biofilms of *P. expansum-A. calcoaceticus.* It should be noticed that dual-species biofilms seem to beneficiate from higher agitation speeds.

Once again, and regarding the scheme proposed by Stepanović et al. (2000) it is possible to verify that after 48 h all cultures, except *A. calcoaceticus* (moderate biofilm producer), were classified as strong biofilm producers. These results propose that one cannnot correlate initial adhesion with the further biofilm formation. In fact, if a microorganism has a low or modest adhesion ability, it does not mean that it is not a strong biofilm producer.

Biofilm interspecies relationships can be established based on the comparison between dual-species biofilm characteristics (ranking) and those from each single biofilm. The existence of synergistic or antagonistic interactions in dual-species biofilm formation was considered whether the biofilm formation category of each single microorganism was smaller or greater, respectively, than that (Simões et al. 2007b). Accordingly, no antagonism in biofilm formation was found and neutral interactions appear to exist in the biofilm formation ability (Table 4 and 5).

3.3.2.2. Metabolic activity assessment

Single and dual-species adherent/biofilm metabolic activity for each time of analysis was assessed using resazurin. Considering the values attained for metabolic activity and productivity for both simple and dual-species biofilms, it is possible to calculate specific metabolic activity values. The results obtained when using 150 rpm as agitation speed for metabolic activity are presented in Figure 4a, and the ones obtained for specific metabolic activity are shown in Figure 4b.

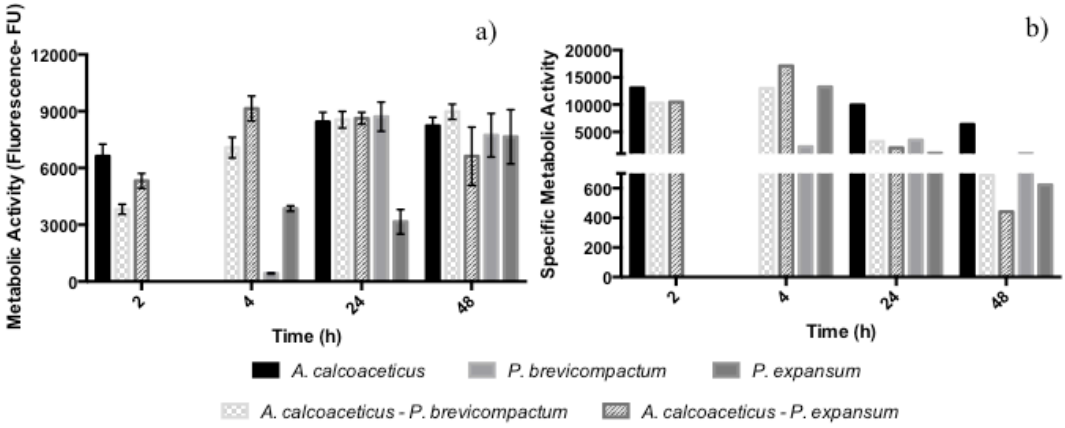

Figure 5- Metabolic activity (a) and specific metabolic activity (b) in terms of fluorescence units as a measure of single and dual-species adherent/biofilm activity overtime for biofilms grown at 150 rpm.

According to the results attained for the metabolic activity assays for single species adhesion of *P. brevicompacum* and *P. expansum*, it is possible to conclude that after a 4 h growth period, *P. expansum* presented reasonably higher values of metabolic activity and specific metabolic activity ($p < 0.05$) when compared with *P. brevicompactum*. Therefore, one can assume that *P. expansum* adapts more easily than *P. brevicompactum*. Nonetheless, after 24 h growth, for *P. brevicompactum,* fairly higher values for metabolic activity were obtained when compared with *P. expansum* ($p < 0.05$), which might indicate that *P. brevicompactum* biofilms are still growing and developing whereas *P. expansum* biofilm could be in a more mature and quasi-stationary state. However, for both fungi, the high values obtained for metabolic activity can be due to the high biomass.

Regarding the values obtained for metabolic activity after 2 h for *A. calcoaceticus* and those obtained for 24 and 48 h it is possible to verify that significantly higher values were obtained for biofilms than for adhered cells ($p < 0.05$). These values might indicate that the biofilm state is more favourable for the bacterium and favours its metabolic activity. However, after analysing the values obtained for specific metabolic activity it is possible to assess that the high values obtained for metabolic activity can be due to the higher amounts of biomass presented by the biofilm. It should be noticed that between 24 and 48 h no significant difference could be perceived for the values obtained for metabolic activity ($p > 0.05$).

After 2 h growth, for the results obtained for metabolic activity of dual-species adhesion of *P. brevicompactum- A. calcoaceticus* and *A. calcoaceticus* single species, it is possible to assess that the bacterium presented significantly higher values for both

metabolic activity and specific metabolic activity ($p < 0.05$). These values might indicate that initially *A. calcoaceticus* alone adapts and grows more easily than its dual-species counterpart. However, after a 24 h period both consortia presented similar values of metabolic activity. Yet, when analysing the results for specific metabolic activity it is possible to assess that the high values obtained for metabolic activity of dual-species biofilms are mainly due to higher amounts of biomass. Additionally, 48 h old dual-species biofilms presented somewhat higher metabolic activity values ($p < 0.05$) than *A. calcoaceticus*, which can once again be explained by the high values of biomass formed. Regarding the results obtained for 4 h growth for both specific and metabolic activity for the aforementioned dual-species and comparing them with those of *P. brevicompactum*, it is possible to assess that the dual-species consortia is more metabolically active than *P. brevicompactum* ($p < 0.05$). These results can indicate that initially the presence of the bacterium promotes adaptation and growth. Notwithstanding, after 24 h growth, both specific and metabolic activities presented no significant differences between the dual and single species biofilms ($p > 0.05$). After 48 h growth, the metabolic activity value was significantly higher for dual-species than for single species biofilms ($p < 0.05$). It should be noticed that for 24 and 48 h the higher values obtained in both consortia, for metabolic activity, might be due to the high amounts of biomass.

Concerning the values obtained for both specific and metabolic activity for dual-species *P. expansum- A. calcoaceticus* and *A. calcoaceticus* grown for 2 h, reasonably higher values were attained for *A. calcoaceticus,* as occurred with the other dual-species consortium. Once again, after 24 h of growth no significant differences can be perceived between the values of metabolic activity obtained for both consortia ($p > 0.05$). Nonetheless, and after analysing the values of specific metabolic activity it is possible to assess the high values obtained for metabolic activity of dual-species biofilms is mostly due to the presence of high amounts of biomass. However, after 48 h growth, significantly higher values of metabolic activity were found for *A. calcoaceticus* than for the *P. expansum- A. calcoaceticus* association ($p < 0.05$). Taking into consideration the values of specific metabolic activity for this time period, one can assess that *A. calcoaceticus* is more metabolic active, which might indicate that the association with *P. expansum* might not be the most beneficial scenario. When doing a similar analysis for *P. expansum- A. calcoaceticus* and *P. expansum* after 4 h, higher values of

metabolic activity can be found for the dual-species consortia ($p < 0.05$). Thus, similar conclusions as those drawn for the other dual-species consortium can be applied here. Moreover, after 24 h fairly higher values of metabolic activity were also attained for the dual-species biofilms ($p < 0.05$) than for single species biofilm of *P. expansum*. Yet, after analysing the specific metabolic activity of the dual-species biofilms, the high values of metabolic activity might be due to high amounts of biomass. Moreover, after 48 h, *P. expansum* biofilms presented higher values for metabolic activity than the dual-species biofilm ($p < 0.05$).

Analysing the results of metabolic activity for both dual-species tested, it is possible to verify that after 2 and 4 h adhesion period, the association of *P. expansum-A.calcoaceticus* shown significantly higher values ($p < 0.05$). These results suggest that the *P. expansum- A. calcoaceticus* association seems to adapt and develop more easily than that of *P. brevicompactum-A. calcoaceticus*. Moreover, for 24 h, both consortia presented similar values for metabolic activity ($p > 0.05$). Additionally, for both dual-species biofilms, it can be suggested that the high values of metabolic activity were due to the high amounts of biomass. After 48 h growth, significantly higher values for metabolic activity were obtained for dual-species biofilms of *P. brevicompactum-A.calcoaceticus* ($p < 0.05$). Also, specific metabolic activity was higher for the *P. brevicompactum-A. calcoaceticus* association, which can indicate that the biofilm is still developing and thriving.

The same assay was performed using 25 rpm as agitation speed. The results obtained when using 25 rpm as agitation speed for metabolic activity are presented in Figure 5a, and those obtained for specific metabolic activity are shown in Figure 5b.

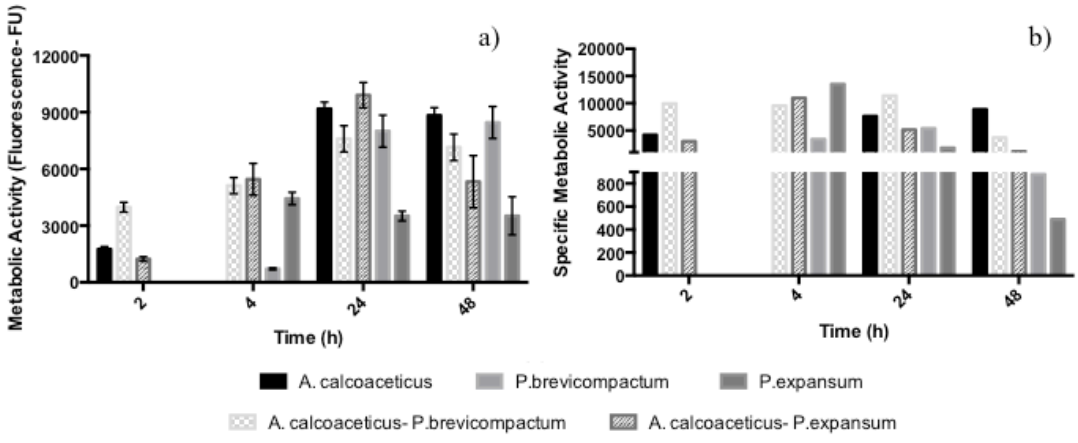

Figure 6- Metabolic activity (a) and specific metabolic activity (b) in terms of fluorescence units as a measure of single and dual-species adherent/biofilm activity overtime for biofilms grown at 25 rpm.

Comparing the values obtained after 4 h for both specific and metabolic activity of *P. brevicompactum* and *P. expansum*, it is possible to verify that significantly higher values were obtained for *P. expansum* ($p > 0.05$), as occurred with 150 rpm as agitation speed. One explanation for these results relies on the fact that perhaps *P. expansum* adapts more easily than *P. brevicompactum*. Notwithstanding, after 24 and 48 h growth *P. brevicompactum* biofilms presented significantly higher values for both specific and metabolic activity ($p < 0.05$), which might be an indication that this fungus thrives under low agitation conditions.

Concerning the values obtained for metabolic activity for 2 h for *A. calcoaceticus* and the ones obtained after 24 and 48 h it is possible to verify that significantly higher values were obtained for biofilms than for adhered cells ($p < 0.05$). Once again, these values might indicate that the biofilm state is more favourable for the bacterium. However, and comparing these results with the ones obtained for 2 hours, but using 150 rpm as agitation speed, significantly smaller values are obtained for 25 rpm ($p < 0.05$), which might indicate that the use of low agitation speeds is not beneficial for bacterial cell viability. For biofilms, after 24 and 48 h no significant difference could be perceived for the values obtained for metabolic activity ($p > 0.05$) between the two hydrodynamics conditions tested.

After 2 h, and concerning the values obtained for both specific and metabolic activity of *P. brevicompactum-A. calcoaceticus* and *A. calcoaceticus,* significantly higher values were obtained for the dual-species sessile cells ($p < 0.05$). These results might indicate that initially and for low agitation speed, the association between *P. brevicompactum* and *A. calcoaceticus* is beneficial, since cells are more active, which can be seen as a marker of active growth (Extremina et al. 2011). Still regarding both consortia, for 24 and 48 h, reasonably higher values for metabolic activity were obtained for *A. calcoaceticus* ($p < 0.05$). Nonetheless, for 24 h, the dual-species consortium presented higher values of specific metabolic activity. As for the comparison between the results obtained for both specific and metabolic activity values of dual-species *P. brevicompactum-A. calcoaceticus* and *P. brevicompactum* after 4 h growth, it is possible to verify, that as occurred for 150 rpm, significantly higher values were obtained for the dual-species association ($p < 0.05$). Hence, the presence of *A. calcoaceticus* might lead to an easier adaption and growth. Nonetheless, after 24 h growth no significant changes between metabolic activities were perceived ($p > 0.05$).

However, for the same period of analysis, higher values for specific metabolic activity were obtained for the dual-species biofilms. Concerning a time period of 48 h, the fungi (*P. brevicompactum*) shows significantly higher biofilm metabolic activity than its dual-species counterpart ($p < 0.05$), which can be explained due to the presence of a high amount of biomass.

As for the results obtained for metabolic activity for 2 h growth for dual-species *P. expansum-A. calcoaceticus* and single species *A. calcoaceticus* sessile cells it is possible to verify that no significant change between these two scenarios were perceived ($p > 0.05$). However, *A. calcoaceticus* presented higher values for specific metabolic activity. For a 24 h growth period, higher metabolic activity values were obtained for dual-species *P. expansum-A. calcoaceticus* biofilms than for single species *A. calcoaceticus* ($p < 0.05$). Nonetheless, taking into consideration the results for specific metabolic activity for this consortium, it is possible to explain the high metabolic activity values due to high amounts of biomass. For a 48 h period, *A. calcoaceticus* presented significantly higher values for metabolic activity than the dual-species biofilms. These results conjugated with the ones obtained for specific activity suggest that the bacterium is more metabolically active and therefore more viable alone than when associated with *P. expansum* under these conditions. Performing a similar analysis for dual-species *P. expansum-A. calcoaceticus* and single species *P. expansum* after 4, 24 and 48 h growth, it is possible to assess that significantly higher values of metabolic activity were obtained for the dual-species association ($p > 0.05$). However, for 24 and 48 h, even though the values for specific metabolic activity are still higher for dual-species *P. expansum-A. calcoaceticus* than those obtained for *P.expansum*, they were lower than the ones obtained for metabolic activity. This difference can be due to the presence of more biomass. Nonetheless, the results imply that the dual-species association seems more active for all times tested under low agitation speed, as occurred for 4 and 24 h when 150 rpm was used as agitation speed.

Concerning the values obtained for both specific and metabolic activity for dual-species *P. brevicompactum-A. calcoaceticus* and *P. expansum- A. calcoaceticus* for a 2 h growth period, it is possible to verify that significantly higher values were obtained for the first dual-species referred ($p < 0.05$), contrarily to what occurred under higher agitation speed. These results suggest that in the initial stages *P. expansum-A. calcoaceticus* association adapts and develops slower than *P. brevicompactum-*

A. calcoaceticus. After 4 h, regarding metabolic activity, there was no significant difference between both consortia ($p > 0.05$). Regarding metabolic activity for 24 h, the association *P. expansum-A. calcoaceticus* presented significantly higher values than *P. brevicompactum-A. calcoaceticus* ($p < 0.05$). Nonetheless, this result can be explained due to higher amounts of biomass. For a period of 48 h, as occurred when 150 rpm were used as agitation speed, the values for metabolic activity were significantly higher for the dual-species *P. brevicompactum-A. calcoaceticus* association. Also, for 48 h, this association presented higher values of specific metabolic activity. Therefore, it is possible to conclude that for both agitation speeds the dual-species biofilms aforementioned seems more active than the *P. expansum-A. calcoaceticus* biofilms.

3.4. Conclusions

A. calcoaceticus and *P. expansum* spores were hydrophilic and *P. brevicompactum* spores were hydrophobic. The fungal surface of *P. brevicompactum* and *P. expansum* were also characterized as hydrophobic using mycelial mats on solid cultures.

Additionally, the results obtained for free energy of adhesion show that *P. expansum* and *P. brevicompactum* are thermodynamically capable of adhering to PS surfaces. However, for *A. calcoaceticus* the adhesion was underestimated since the adhesion to PS surface was possible.

Regarding the adhesion and biofilm formation assays, it was possible to assess that, for both hydrodynamic conditions tested the dual-species biofilms of *P. expansum-A. calcoaceticus* presented higher productivity than the biofilms formed by *P. brevicompactum-A. calcoaceticus* association. However, for mature biofilms (48 h), the association between *P. brevicompactum-A. calcoaceticus* proved to be more metabolically active than those previously mentioned.

Also, it can be concluded that *P. brevicompactum* thrives when grown under low agitation speeds (25 rpm) due to the high values obtained for biomass quantification and metabolic activity. Moreover, under low agitation speed, fungal single species biofilms presented higher amounts of biomass than the dual-species counterparts. The association between the fungi and *A. calcoaceticus* does not seem to present any great disadvantage for either species and no antagonism can be perceived for the biofilms (24 and 48 h old) tested.

Chapter 4

4. Control of single- and dual-species biofilms of *Acinetobacter calcoaceticus*, *Penicillium expansum*, *Penicillium brevicompactum* with sodium hypochlorite

4.1. Introduction

Living in the sessile state confers the microorganisms several advantages when compared with cells in the planktonic state. One of those advantages is the fact that the biofilm mode of growth enables resistance to a number of control strategies, particularly antimicrobial and mechanical stresses (Bridier et al. 2011). In a DWDS, disinfection with chlorine and chlorine dioxide can reduce the concentration of planktonic bacteria, but have almost no effect on the concentration of biofilm bacteria (Simões et al. 2010b).

Currently there are not many studies about ff biofilms when compared with bacteria and yeast, which have been extensively studied (Huq et al. 2008). Nonetheless, it should be noticed that ff are especially adapted for surface growth due to their absorptive nutrition mode, their secretion of extracellular enzymes to digest complex molecules, and apical hyphal growth (Elvers et al. 2001). Furthermore, there are several reports of the presence of ff in DWDS worldwide (Hageskal et al. 2009).

From all the chemical disinfectants used for DWDS treatment, chlorine is the most commonly used. The chemical disinfection associated with the maintenance of chlorine residual concentration through the distribution systems is a very popular strategy to prevent the regrowth of microorganisms during water transportation (Simões 2013). Chlorine mode of action relies on the fact that it can cause physiological damage to the cell membrane. This damage is due to the fact that cytochromes, iron-sulphur proteins and nucleotides are extremely vulnerable to the chlorine oxidative effect. Therefore, after exposure of the cells to chlorine treatment, several major cell functions like respiration, glucose transport and ATP levels present lower values (WHO 2011). However, the presence of residual chlorine is one of the stress factors that can cause biofilm formation (Kokare & Chakraborty 2009).

DWDS are environments where different microorganisms cohabit and interact, living mainly under the sessile state (Davey et al. 2000). Therefore, multispecies

biofilms are progressively recognized as an important phenomenon. However, their properties remain poorly understood, and there is few data on disinfectant susceptibility of multispecies biofilms (Kart et al. 2014). Many of these studies regard only multispecies biofilms of bacteria (Schwering et al. 2013; Simões et al. 2010b), which is not a realistic scenario. Thus, studies on multispecies biofilms comprised of both fungi and bacteria, especially ff, are of extreme importance so that more effective disinfection systems can be developed.

In this chapter both single and dual-species mature biofilms (48 h) developed at two different hydrodynamic conditions were exposed to a disinfection treatment using different concentrations (0; 0.1; 0.5; 1; 10 and 100 mg/L) of SHC. The effects of the different concentrations of disinfectant were used to inactivate and remove single and dual-species biofilms formed by the selected microorganisms.

4.2. Materials and methods

4.2.1. Microorganisms and culture conditions

The microorganisms and culture conditions used for biofilm development are described in Section 3.2.1.

4.2.2. Biofilm disinfection assay

Single and dual-species biofilms were developed during 48 h as described in section 3.2.5. at agitation speeds of 150 and 25 rpm.

Disinfection treatment of biofilms was performed according to Simões et al. (2010b). Briefly, after the biofilm formation period, the content of each well was removed and the wells were washed three times with 250 mL of sterile distilled water to remove reversibly adherent microorganisms. This way, only the remaining attached microorganisms on the inner walls of the wells were submitted to the disinfection assay. A stock solution of SHC was prepared by diluting a commercially available solution (Sigma, Portugal) with sterile distilled water. The disinfectant solutions with the various concentrations used in this assay (0.1, 0.5, 1, 10 and 100 mg/L) were prepared on the day of use, under aseptic conditions, and stored in the dark at 4°C. Immediately after rinsing, the biofilms were exposed to the different SHC concentrations. At least 16 wells of the 96-wells microtiter plate were filled with 250 μL of each concentration of SHC. Additionally, untreated biofilm wells (control) were also used for each condition

tested. The SHC solutions remained in contact with the biofilms for 1 h, being refreshed every 30 min during the 1 h treatment period. The refreshment of the disinfectant solutions occurred since the biofilms have a high density of cells, and low volumes of SHC solutions are being applied for treatment (Shakeri et al. 2007). During the disinfection treatment the microtiter plates were incubated on a shaker at 150 rpm and at 25 ºC, in order to increase the contact of biofilm cells with the disinfectant. Post treatment, the disinfectant solutions were removed and the wells were rinsed twice with 250 µL of sterile sodium thiosulfate solution (VWR, Portugal) at 0.5% (wt/vol) to quench the activity of the disinfectant. Additionally, the wells were rinsed one time with 250 µL of sterile distilled water.

4.2.3. Metabolic activity assessment using alamar blue/resazurin

Single and dual-species biofilm inactivation was analysed through metabolic activity assessment using resazurin as described in section 3.2.6.

The results obtained were expressed as biofilm inactivation percentage calculated using the following expression:

$$Biofilm\ inactivation\ (\%) = \frac{(C-B)-(T-B)}{(C-B)} \times 100 \quad (4)$$

where B indicates the average fluorescence for the blank wells (without microorganism), C indicates the average fluorescence for the control wells (untreated biofilms), and T indicates the average fluorescence for the SHC-treated wells.

4.2.4. Biomass quantification using crystal violet

Single and dual-species biofilm removal was assessed by biomass quantification using CV as described in section 3.2.7.

The results obtained were expressed as biofilm mass removal percentage calculated using the following expression:

$$Biofilm\ mass\ removal\ (\%) = \frac{(C-B)-(T-B)}{(C-B)} \times 100 \quad (5)$$

where B indicates the average absorbance for the blank wells (without microorganism), C indicates the average absorbance for the control wells (untreated biofilms), and T indicates the average absorbance for the SHC-treated wells.

4.2.5. Statistical analysis

The data was analysed using One-Way Anova. Significance level for the difference between data was set at $p < 0.05$. The data was analysed using the statistical program Prism 6. It should be noticed that for each condition tested three replicates were performed.

4.3. Results and discussion

. In order to better understand the role of ff and BFIs on DW biofilm control by SHC, a commonly used disinfectant in DWDS, the effects of SHC on single and dual-species (bacterial and ff) biofilms and their impact on biofilm inactivation and on biofilm removal from PS surface was studied. According to my knowledge there are no published reports on this subject. In this assays were used matured biofilms (48 h) grown in PS microtiter plates at different hydrodynamic conditions (150 and 25 rpm) for all the conditions tested.

The results attained for the disinfection treatment using single and dual-species biofilms grown at 150 rpm are shown in Tables 6 and 7. Table 6 presents the percentage of biofilm metabolic inactivation and Table 7 shows the percentage of biofilm mass removal.

Appendix D provides full Tables (Table D.1 ,2 ,3 and 4) with both metabolic activity and biomass quantification values.

Table 6- Results of the percentage of biofilm inactivation for single and dual-species biofilms at 150 rpm with several SHC concentrations. The values with * were not calculated/considered since the metabolic activity values were higher or equal to the control (0 mg/L)

SHC (mg/L)	Biofilm inactivation (%)				
	P. expansum	***P. brevicompactum***	***A. calcoaceticus***	***P. expansum-A. calcoaceticus***	***P. brevicompactum-A. calcoaceticus***
0.1	22	9	2	6	2
0.5	53	5	16	1	0 *
1	37	3	45	5	0 *
10	47	1	96	7	21
100	25	0 *	97	54	92

Table 7- Results of the percentage of biofilm mass removal for single and dual-species biofilms at 150 rpm with several SHC concentrations

SHC (mg/L)	Biofilm mass removal (%)				
	P. expansum	***P. brevicompactum***	***A. calcoaceticus***	***P. expansum-A. calcoaceticus***	***P. brevicompactum-A. calcoaceticus***
0.1	22	5	37	34	1
0.5	24	12	36	32	11
1	33	1	38	41	13
10	36	12	66	47	34
100	39	15	74	6	41

Comparing the effectiveness of disinfection between single species biofilm of *P. expansum* and *P. brevicompactum*, higher values of biofilm inactivation and biofilm removal percentages were attained for *P. expansum*. These results suggest that 48 h biofilms of *P. brevicompactum* are more resistant than the ones formed by *P. expansum*. For both fungi, it is possible to verify that when high concentrations of SHC were used the values of the percentage of metabolic activity and biofilm removal were lower than what could be expected.

Regarding the dual-species biofilms formed by *P. expansum-A. calcoaceticus,* it is possible to verify that, in general, smaller values of biofilm inactivation percentage were achieved when compared with both single species biofilms of *P. expansum* and *A. calcoaceticus*. These results corroborate previous findings where multispecies biofilms of bacteria appeared to be more resistant than the single species ones (Lindsay et al. 2002; Simões et al. 2009; Simões et al. 2010b). Notwithstanding, the dual-species biofilm presented higher biomass removal percentages than single species biofilms of *P. expansum*.

Dual-species biofilms of *P. brevicompactum-A. calcoaceticus* presented high values of biofilm inactivation percentage for higher concentrations of SHC, meaning in this case that inactivation was concentration dependent. Furthermore, for concentrations of 10 and 100 mg/L the dual-species biofilm appeared to be less resistant than single species biofilm of *P. brevicompactum*. Also, the dual-species biofilms appeared to be more resistant than the biofilm formed by single species *A. calcoaceticus* since the values obtained for both biofilm inactivation and biofilm mass removal percentages were smaller. Once again, biofilm mass removal percentages were higher for the dual-species biofilms when compared with single species biofilms formed by

P. brevicompactum. Thus a similar behaviour of both dual-species biofilms seems to occur.

The results obtained for the biofilm inactivation percentage demonstrated that dual-species biofilms of *P. brevicompactum-A. calcoaceticus* suffered higher inactivation than those formed by *P. expansum-A. calcoaceticus,* when exposed to high concentrations of SHC (10 and 100 mg/L), which can indicate that these dual-species biofilms are less resistant to disinfection. However, and taking into consideration the results obtained for biofilm mass removal percentage it is possible to assess that the biofilms formed by *P. expansum-A. calcoaceticus* suffered for almost all disinfectant concentrations (except the higher tested), higher biomass removals than the biofilms formed by *P. brevicompactum-A. calcoaceticus*. These results might be an indication that the association between *A. calcoaceticus* and the different fungi will lead to biofilms with different susceptibilities to SHC. Furthermore, and still comparing the two dual-species biofilms, it appears that the one formed by *P. expansum-A. calcoaceticus* (under high agitation speeds) was less cohesive than the one formed by *P. brevicompactum-A. calcoaceticus*.

The control of single and dual-species biofilms with SHC was also performed for biofilms grown under low agitation speeds (25 rpm). Table 8 presents the percentage of biofilm inactivation and Table 9 shows the percentage of biofilm mass removal.

Table 8- Results of percentage of biofilm inactivation for single and dual-species biofilms at 25 rpm with several SCH concentrations. The values with * were not calculated/considered since the metabolic activity values are higher or equal to the control (0 mg/L)

SHC (mg/L)	**Biofilm inactivation (%)**				
	P. expansum	***P. brevicompactum***	***A. calcoaceticus***	***P. expansum-A. calcoaceticus***	***P. brevicompactum-A. calcoaceticus***
0.1	51	0 *	17	8	0 *
0.5	60	0 *	18	13	0 *
1	64	1	29	13	0 *
10	91	0 *	93	41	14
100	46	0 *	93	96	96

Table 9- Results of the percentage of biofilm mass removal for single and dual-species biofilms at 25 rpm with several SHC concentrations. The values with * were not calculated/considered since the metabolic activity values are higher or equal to the control (0 mg/L)

SHC (mg/L)	Biofilm mass removal (%)				
	P. expansum	***P. brevicompactum***	***A. calcoaceticus***	***P. expansum-A. calcoaceticus***	***P. brevicompactum-A. calcoaceticus***
0.1	30	0 *	29	21	18
0.5	35	0 *	30	43	0 *
1	41	0 *	19	3	0 *
10	44	0 *	60	0 *	8
100	36	0 *	77	0 *	3

Analysing the results obtained for single species biofilms of *P. expansum* and *P. brevicompactum,* in terms of biofilm metabolic inactivation (Table 8) it is possible to ascertain that *P. expansum* appears to be more susceptible to disinfection. It should be noticed that no significant difference could be perceived between the values of metabolic activity for all disinfectant concentrations used for *P. brevicompactum* ($p > 0.05$) (data shown in table D.3 from appendix section D). Thus, this might mean that biofilms of *P. brevicompactum* grown under low agitation speeds are extremely resistant to disinfection. Also, for the percentage of biomass removal (data shown in table D.4 from the appendix section D), no significant difference can be perceived for concentration up to 1 mg/L of SHC ($p > 0.05$). However, for concentrations of 10 and 100 mg/L of SHC, significant differences were perceived between the values obtained and the control ($p < 0.05$). The possible explanation for this might rely on the fact that the presence of high concentrations of disinfectant can damage the biofilm structure allowing an easier access to the fungi structures by CV dye.

Regarding the results obtained for biofilm inactivation percentage (Table 8) for dual-species biofilms of *P. expansum-A. calcoaceticus* and comparing them with the ones obtained for single species biofilms of *P. expansum*, it is possible to assess that the dual-species biofilms suffer less inactivation and, therefore, can be perceived as more resistant for all the disinfectant concentration tested except for SHC of 100 mg/L to disinfection. For this SHC concentration, *P. expansum* biofilms showed lower inactivation values than expected. Regarding the results of the percentage of biofilm mass removal (Table 9) it is possible to verify that single species biofilms of *P. expansum* suffered higher biomass removals than the dual-species for all SHC

concentrations with exception of 0.5 mg/L of SHC. The high biomass amounts of the dual-species biofilm after treatment with 10 and 100 mg/L is probably due to the damage of the biofilm structure, which will allow an easier penetration of the dye into the cells matrix. Dual-species biofilms of *P. expansum-A. calcoaceticus* also appeared to be more resistant than single species biofilm of *A. calcoaceticus*, as occurred for this biofilm at 150 rpm. This conclusion can be drawn since lower values for both biofilm inactivation and biofilm mass removal percentages were obtained for the dual-species biofilms for all disinfectant concentrations used up to 10 mg/L. For the treatment using 100 mg/L of SHC it is possible to assess that the dual-species biofilms presented higher inactivation values than the single species biofilms of *A. calcoaceticus.*

Concerning the values for biofilm inactivation percentage obtained for the dual-species biofilms of *P. brevicopactum-A. calcoaceticus*, it is possible to assess that for high SHC concentration (10 and 100 mg/L), inactivation occurred and increased with the concentration of disinfectant. Also, the values obtained for metabolic activity (data shown in Table D.3 from the Appendix section D) of the biofilm after treatment for concentrations up to 1 mg/L of SHC presented no significant difference when compared with the control/SHC-untreated biofilms ($p > 0.05$), which can indicate that lower concentrations of disinfectant had little or no effect on the biofilm metabolic activity. Additionally, the results obtained for biofilm mass quantification (data shown in Table D.4 from the Appendix section D) for all conditions tested presented no significant difference when compared with the control assays ($p > 0.05$). So, the percentages possible to calculate (0.1, 10 and 100 mg/L) are also an indication of the low effectiveness of the disinfection. Nonetheless, and taking into consideration the results obtained for both test for single species biofilms of *P. brevicompactum*, one can assume that dual-species biofilms are less resistant than the single species biofilms regardless of the agitation condition under which are grown. Furthermore, single species biofilms of *A. calcoaceticus* also appeared to be more susceptible to disinfection than dual-species biofilms of *P. brevicompactum-A. calcoaceticus*, due to the higher values obtained for both biofilm inactivation and biofilm mass removal percentages.

Comparing both dual-species biofilms the one composed by *P. expansum-A. calcoaceticus* presented some inactivation caused by the treatment when concentrations up to 0.5 mg/L SHC were used. Furthermore, and comparing both biofilm inactivation percentage obtained for the two biofilms when a SHC solution of 10 mg/L was applied, it is possible to verify that the *P. expansum-A. calcoaceticus* were

more susceptible to inactivation by this disinfectant concentration. Also, for treatments using 100 mg/L the same inactivation percentage was obtained for both biofilms. Thus, it seems that dual-species biofilms of *P. expansum-A. calcoaceticus* are more predisposed to inactivation cause by SHC.

It should be noticed that there are some unexpected incongruences and values' oscillations that are hard to rationalize and no simple explanation can be drawn. Furthermore, the methodology used is composed by expedite methods that can be applied both to bacteria and fungi and were not optimized for the association of the two types of microorganisms. Moreover, when using resazurin it is necessary to consider that different microorganism will metabolize differently the stain, imposing the necessity to establish the optimal dye incubation time in each case (Sandberg et al. 2009), which is not possible for this type of analysis.

4.4. Conclusions

Biofilms of *P. brevicompactum* grown under low and high agitation speed appeared to be more resistant to disinfection using SHC than the other single and dual-species biofilms tested.

Dual-species biofilms of *P. expansum-A. calcoaceticus* suffered less inactivation than single species biofilms of *P. expansum*, which suggests that the presence of *A. calcoaceticus* confers resistance to the biofilm. Conversely, biofilms of *P. brevicompactum-A. calcoaceticus* suffered higher inactivation than single species biofilms of *P. brevicompactum,* which indicates that biofilms of *P. brevicompactum* are extremely resistant and the association with *A. calcoaceticus* is not beneficial for antimicrobial resistance for 150 rpm.

The results obtained for the dual-species biofilms of *P. brevicompactum-A. calcoaceticus* suggest that these biofilms are more easily inactivated than dual-species biofilms of *P. expansum- A. calcoaceticus* for 150 rpm.

For *A. calcoaceticus* the association with both fungi seems to be beneficial for its antimicrobial resistance when compared with the results obtained for its single species biofilms.

Chapter 5

5. Final remarks and research needs

5.1. General conclusions

Although some reports have been made regarding the presence of fungi and ff in biofilms, in DW their role in them is yet not fully understood. The main reason for this is the fact that so far the presence of fungi in DW has not caused any acute symptoms and disease in humans. Thus, the studies performed during this dissertation are of importance since they bring to light relatively new aspects regarding the behaviour of two ff and one bacterium isolated from DW.

It was possible to characterize the bacterium surface as being hydrophilic. A novel technique was used to characterize the surface of *P. brevicompactum* and *P. expansum* spores as hydrophobic and hydrophilic, respectively, even if not apparently reliable for *P. expansum*. Nevertheless, and regarding the theoretical prevision of adhesion, it was possible to assess that the previsions for the ff spores were correlated with *in vitro* adhesion experiments. In fact, adhesion occurred. However, for *A. calcoaceticus* the prevision underestimated the bacterium capability to adhere to PS and *in vitro* assays showed that adhesion was possible. Additionally, fungal colonies surface were also characterized and showed that both fungi, in different zones of the colony (different ages of fungi), were always hydrophobic. For both agitation speeds tested (25 and 150 rpm), dual-species biofilms and single species biofilms of fungi presented high metabolic activity, which was mainly related to the presence of high amounts of biomass. Additionally, for dual species-biofilms productivity appears to be higher when the agitation speed is bigger. Moreover, the association between the ff studied and *A. calcoaceticus* does not seem to present a great disadvantage or advantage in biofilm productivity and activity for either species and no antagonism was perceived for the 24 and 48 h old biofilms.

Mature biofilms (48 h) of *P. brevicompactum* are extremely resistant to disinfection since no inactivation or removal could be achieved upon treatment with different concentrations. Furthermore, and for this ff, the association with A. calcoaceticus was not advantageous in terms of biofilm control with SHC for the fungi. Conversely, the presence of *A. calcoaceticus* in biofilms with *P. expansum* seems

to beneficiate the ff since these biofilms appears to be more resistant. Also, for *A. calcoaceticus*, the association with these ff appears to be helpful in the presence of disinfectant, since single species biofilms of the bacterium were highly susceptible when compared with the dual-species biofilms. Generally, dual-species biofilms grown under high agitation speeds (150 rpm) appear to suffer higher biomass removal.

5.2. Future work

As previously mentioned there is still a lot of work to be performed when discussing the presence of ff and their role in DW. Thus, and to completely verify the conclusions obtained during the development of this dissertation, further studies need to be done in order to better characterize the biofilms formed and understand their differences.

Some doubts about the performance of the disinfection treatment performed in both inactivation and biofilm removal remained after the tests performed. These doubts are mainly caused by the oscillation of the values, which can be due to the use of expedite methods that were not optimized for the microorganism nor the experiment in question. Hence, other methods could be analysed and applied if found more adequate. Furthermore, and considering that many of the biofilms tested showed low inactivation and removal, other commonly used chemical disinfectants could be tested in order to verify their efficacy. Additionally, mechanical treatments could also be applied in parallel with the chemical agent to potentiate the biofilm control.

Appropriate methodologies of analysis for both ff and bacteria need to be found, in order to prevent incongruences and errors. For example, when using resazurin an optimal dye incubation period needs to be established and this time will vary according with the microorganisms used (Sandberg et al. 2009). The establishment of such methodologies will be very helpful since normally the data collected in different laboratories cannot be compared most of the times, due to lack of standardised and adequate methodology (Gonçalves et al. 2006a , Hageskal et al. 2009).

References

Allison, D.G., Sutherland, I.W., 1987. The role of exopolysaccharides in adhesion of freshwater bacteria. *Journal of General Microbiology*, 133, pp.1319–1327.

Amiri, A., Cholodowski, D., Bompeix, G., 2005. Adhesion and germination of waterborne and airborne conidia of *Penicillium expansum* to apple and inert surfaces. *Physiological and Molecular Plant Pathology*, 67(1), pp.40–48.

An, Y.H., Friedman, R.J., 1998. Concise review of mechanisms of bacterial adhesion to biomaterial surfaces. *Journal of Biomedical Materials Research*, 43(3), pp.338–348.

Anaissie, E.J., Stratton, S.L., Dignani, M.C., Lee, C., Summerbell, R.C., Rex, J.H., Monson, T.P., Walsh, T.J., 2003. Pathogenic molds (including *Aspergillus* species) in hospital water distribution systems: a 3-year prospective study and clinical implications for patients with hematologic malignancies. *Blood*, 101(7), pp.2542–2546.

Bayram, O., Braus, G.H., 2012. Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. *FEMS Microbiology Reviews*, 36(1), pp.1–24.

Beaudeau, P., de Valk, H., Vaillant, V., Mannschott, C., Tillier, C., Mouly, D., Ledrans, M., 2008. Lessons learned from ten investigations of waterborne gastroenteritis outbreaks, France, 1998-2006. *Journal of Water and Health*, 6(4), pp.491–503.

Beauvais, A., Schmidt, C., Guadagnini, S., Roux, P., Perret, E., Henry, C., Paris, S., Mallet, A., Prévost, M.-C., Latgé, J.P., 2007. An extracellular matrix glues together the aerial-grown hyphae of *Aspergillus fumigatus*. *Cellular Microbiology*, 9(6), pp.1588–1600.

Berry, D., Xi, C., Raskin, L., 2006. Microbial ecology of drinking water distribution systems. *Current Opinion in Biotechnology*, 17(3), pp.297–302.

Bos, R., van der Mei, H.C., Busscher, H.J., 1999. Physico-chemistry of initial microbial adhesive interactions-its mechanisms and methods for study. *FEMS Microbiology Reviews*, 23(2), pp.179–230.

Bridier, A., Briandet, R., Thomas, V., Dubois-Brissonnet, F., 2011. Resistance of bacterial biofilms to disinfectants: a review. *Biofouling*, 27(9), pp.1017–1032.

Bryers, J.D., Ratner, B.D., 2004. Bioinspired implant materials befuddle bacteria. *ASM News*, 70(5), pp.232–237.

Busscher, H.J., Bos, R., van der Mei, H.C., 1995. Initial microbial adhesion is a determinant for the strength of biofilm adhesion. *FEMS Microbiology Letters*, 128(3), pp.229–234.

Chandy, J.P., Angles, M.L., 2001. Determination of nutrients limiting biofilm formation and the subsequent impact on disinfectant decay. *Water Research*, 35(11), pp.2677–2682.

Chau, H.W., Si, B.C., Goh, K.Y., Vujanovic, V., 2009. A novel method for identifying hydrophobicity on fungal surfaces. *Mycological Research*, 113(Pt 10), pp.1046–1052.

Chau, H.W., Goh, K.Y., Si, B.C., Vujanovic, V., 2010. Assessment of alcohol percentage test for fungal surface hydrophobicity measurement. *Letters in Applied Microbiology*, 50(3), pp.295–300.

Chmielewski, R.A.N., Frank, J.F., 2003. Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety*, 2(1), pp.22–32.

Chowdhury, S., 2012. Heterotrophic bacteria in drinking water distribution system: a review. *Environmental Monitoring and Assessement,* 184(10), pp. 6087-6137

Davey, M.E., George, A.O., O'Toole, G.A., 2000. Microbial biofilms : from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews*, 64(4), pp.847–867.

Deborde, M., von Gunten, U., 2008. Reactions of chlorine with inorganic and organic compounds during water treatment-Kinetics and mechanisms: a critical review. *Water Research*, 42(1-2), pp.13–51.

De Gusseme, B., Vanhaecke, L., Verstraete, W., Boon, N., 2011. Degradation of acetaminophen by *Delftia tsuruhatensis* and *Pseudomonas aeruginosa* in a membrane bioreactor. *Water Research*, 45(4), pp.1829–1837.

Doggett, M.S., 2000. Characterization of fungal biofilms within a municipal water distribution system. *Applied and Environmental Microbiology*, 66(3), pp.11–14.

Donlan, R.M., 2002. Biofilms: microbial life on surfaces. *Emerging Infectious Diseases*, 8(9), pp.881–890.

Donlan, R.M., Costerton, J., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*, 15(2), pp.167–193.

Doyle, R.J., 2000. Contribution of the hydrophobic effect to microbial infection. *Microbes and Infection*, 2(4), pp.391–400.

Elvers, K.T., Leeming, K., Lappin-Scott, H.M., 2001. Binary culture biofilm formation by *Stenotrophomonas maltophilia* and *Fusarium oxysporum*. *Journal of Industrial Microbiology & Biotechnology*, 26(3), pp.178–183.

Elvers, K.T., Leeming, K., Moore, C.P., Lappin-Scott, H.M., 1998. Bacterial-fungal biofilms in flowing water photo-processing tanks. *Journal of Applied Microbiology*, 84(4), pp.607–618.

Extremina, C.I., Costa, L., Aguiar, A.I., Peixe, L., Fonseca, A.P., 2011. Optimization of processing conditions for the quantification of enterococci biofilms using microtitre-plates. *Journal of Microbiological Methods*, 84(2), pp.167–173.

Farkas, A., Dragan-Bularda, M., Muntean, V., Ciataras, D., Tigan, S., 2012. Microbial activity in drinking water-associated biofilms. *Central European Journal of Biology*, 8(2), pp.201–214.

Frey-Klett, P., Burlinson, P., Deveau, A., Barret, M., Tarkka, M., Sarniguet, A., 2011. Bacterial-fungal interactions: hyphens between agricultural, clinical, environmental, and food microbiologists. *Microbiology and Molecular Biology Reviews*, 75(4), pp.583–609.

Gilbert, N., 2012. Water under pressure. *Nature*, 483(7389), pp.256–257.

Gomes, I.B., Simões, M., Simões, L.C., 2014. An Overview on the reactors to study drinking water biofilms. *Water Research*, 62, pp.63–87.

Gonçalves, A.B., Paterson, R.R.M., Lima, N., 2006a. Survey and significance of filamentous fungi from tap water. *International journal of hygiene and Environmental Health*, 209(3), pp.257–264.

Gonçalves, A.B., Santos, I.M., Paterson, R.R.M., Lima, N., 2006b. FISH and calcofluor staining techniques to detect in situ filamentous fungal biofilms in water. *Revista Iberoamericana de Micologia*, 23, pp.194–198.

Grabińska-Łoniewska, A., Konieczkowicz-Kowalska, T., Wardzyńska, G., Boryn, K., 2007. Occurrence of fungi in water distribution system. *Polish Journal of Environmental Studies*, 16(4), pp.539–547.

Gutiérrez-Correa, M., Villena, G., 2003. Surface adhesion fermentation : a new fermentation category. *Revista Peruana de Biología*, 10(2), pp.113–124.

Hageskal, G., Gaustad, P., Heier, B.T., Skaar, I., 2007. Occurrence of moulds in drinking water. *Journal of Applied Microbiology*, 102(3), pp.774–780.

Hageskal, G., Lima, N., Skaar, I., 2009. The study of fungi in drinking water. *Mycological Research*, 113(Pt 2), pp.165–172.

Harding, M.W., Marques, L.L.R., Howard, R.J., Olson, M.E., 2009. Can filamentous fungi form biofilms?. *Trends in Microbiology*, 17(11), pp.475–480.

Hayette, M.P., Christiaens, G., Mutsers, J., Barbier, C., Huynen, P., Melin, P., de Mol, P., 2010. Filamentous fungi recovered from the water distribution system of a Belgian university hospital. *Medical Mycology*, 48(7), pp.969–974.

Huq, A., Whitehouse, C.A., Grim, C.J., Alam, M., Colwell, R.R., 2008. Biofilms in water, its role and impact in human disease transmission. *Current Opinion in Biotechnology*, 19(3), pp.244–247.

Janczuk, B., Chibowski, E., Bruque, J.M., Kerkeb, M.L., González Caballero, F.,1993. On the consistency of surface free energy components as calculated from contact angles of different liquids- An application to the cholesterol surface. *Journal of Colloid and Interface Science*, 159(2), pp.421–428.

Jones, E.B.G., 1994. Fungal adhesion. *Mycological Research*, 98(9), pp.961–981.

Kart, D., Tavernier, S., van Acker, H., Nelis, H.J., Coenye, T., 2014. Activity of disinfectants against multispecies biofilms formed by *Staphylococcus aureus*, *Candida albicans* and *Pseudomonas aeruginosa*. *Biofouling*, 30(3), pp.377–383.

Kim, S.D., Cho, J., Kim, I.S., Vanderford, B.J., Snyder, S.A., 2007. Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. *Water Research*, 41(5), pp.1013–1021.

Kobayashi, D.Y., Crouch, J.A., 2009. Bacterial/fungal interactions: from pathogens to mutualistic endosymbionts. *Annual Review of Phytopathology*, 47, pp.63–82.

Kokare, C., Chakraborty, S., 2009. Biofilm: Importance and applications. *Indian Journal of Biotechonology*, 8, pp.159–168.

Liberti, L., Notarnicola, M., Petruzzelli, D., 2003. Advanced treatment for municipal wastewater reuse in agriculture. UV disinfection: parasite removal and by-product formation. *Desalination*, 152, pp.315–324.

Linder, M.B., Szilvay, G.R., Nakari-Setälä, T., Penttilä, M.E., 2005. Hydrophobins: the protein-amphiphiles of filamentous fungi. *FEMS Microbiology Reviews*, 29(5), pp.877–896.

Lindsay, D., Brözel, V.S., Mostert, J.F., van Holy, A., 2002. Differential efficacy of a chlorine dioxide-containing sanitizer against single species and binary biofilms of

a dairy-associated *Bacillus cereus* and a *Pseudomonas fluorescens* isolate. *Journal of Applied Microbiology*, 92(2), pp.352–361.

Momba, M., Kfir, R., Venter, S., Cloete, T., 2000. An overview of biofilm formation in distribution systems and its impact on the deterioration of water quality. *Water SA*, 26(1), pp.59–66.

Morales, D.K., Grahl, N., Okegbe, C., Dietrich, L.E.P, Jacobs, N.J., Hogan, D.A., 2010. *Candida albicans* interactions with bacteria in the context of human health and disease. *mBio*, 6(4), pp.1–9.

Mowat, E., Butcher, J., Lang, S., Williams, C., Ramage, G., 2007. Development of a simple model for studying the effects of antifungal agents on multicellular communities of *Aspergillus fumigatus*. *Journal of Medical Microbiology*, 56(Pt 9), pp.1205–1212.

Mowat, E., Williams, C., Jones, B., McChlery, S., Ramage, G., 2009. The characteristics of *Aspergillus fumigatus* mycetoma development: is this a biofilm? *Medical mycology : official publication of the International Society for Human and Animal Mycology*, 47(1), pp.S120–S126.

Nieuwenhuijsen, M.J., Toledano, M.B., Eaton, N.E., Fawell, J., Elliott, P., 2000. Chlorination disinfection byproducts in water and their association with adverse reproductive outcomes: a review. *Occupational and Environmental Medicine*, 57(2), pp.73–85.

O'Toole, G., Kaplan, H., Kolter, R., 2000. Biofilm formation as microbial development. *Annual Reviews in Microbiology*, 54, pp.49–79.

van Oss, C.J., 1997. Hydrophobicity and hydrophilicity of biosurfaces. *Current Opinion in Colloid & Interface Science*, 2(5), pp.503–512.

van Oss, C. J., Chaudhury, M. K., Good, R. J., 1987. Monopolar surfaces. *Advances in Colloid and Interface Science,* 28, pp 35-64.

van Oss, C. J., Good, R. J., Chaudhury, M. K., 1988. Additive and nonadditive surface tension components and the interpretation of contact angles. *Langmuir,* 4(4), pp 884-891.

van Oss, C. J., Ju, L., Chaudhury, M. K., Good, R. J., 1989. Estimation of the polar parameters of the surface tension of liquids by contact angle measurements on gels. *Journal of Colloid and Interface Science,*128(2), pp 313-319.

Pascual, S., De Cal, A., Magan, N., Melgarejo, P. 2000. Surface hydrophobicity,

viability and efficacy in biological control of *Penicillium oxalicum* spores produced in aerial and submerged culture. *Journal of Applied Microbiology*, 89(5), pp.847–853.

Paterson, R., Lima, N., 2005. Fungal contamination of drinking water. In J. Lehr et al., eds. *Water Encyclopedia*. John Wiley & Sons, Inc.

Pavithra, D., Doble, M., 2008. Biofilm formation, bacterial adhesion and host response on polymeric implants-issues and prevention. *Biomedical Materials*, 3(3), pp. 1–13.

Pereira, V.J., Basílio, M.C., Fernandes, D., Domingues, M., Paiva, J.M., Benoliel, M.J., et al., 2009. Occurrence of filamentous fungi and yeasts in three different drinking water sources. *Water Research*, 43(15), pp.3813–3819.

Peters, B.M., Jabra-Rizk, M.A., Scheper, M.A., Leid, J.G., Costerton, J.W., Shirtliff, M.E., 2010. Microbial interactions and differential protein expression in *Staphylococcus aureus-Candida albicans* dual-species biofilms. *FEMS Immunology and Medical Microbiology*, 59(3), pp.493–503.

Rand, J.L., Hofmann, R., Alam, M.Z.B., Chauret, C., Cantwell, R., Andrews, R.C., Gagnon, G.A., 2007. A field study evaluation for mitigating biofouling with chlorine dioxide or chlorine integrated with UV disinfection. *Water Research*, 41(9), pp.1939–1948.

Rateb, M.E., Hallyburton, I., Houssen, W.E., Bull, A.T., Goodfellow, M., Santhanam, R., Jaspars, M., Ebel, R., 2013. Induction of diverse secondary metabolites in *Aspergillus fumigatus* by microbial co-culture. *RSC Advances*, 3(34), p.14444–14450.

Reverberi, M., Ricelli, A., Zjalic, S., Fabbri, A.A., Fanelli, C., 2010. Natural functions of mycotoxins and control of their biosynthesis in fungi. *Applied Microbiology and Biotechnology*, 87(3), pp.899–911.

Sandberg, M.E., Schellmann, D., Brunhofer, G., Erker, T., Busygin, I., Leino, R., Vuorela, P.M., Fallarero, A., 2009. Pros and cons of using resazurin staining for quantification of viable *Staphylococcus aureus* biofilms in a screening assay. *Journal of Microbiological Methods*, 78(1), pp.104–106.

Schwering, M., Song, J., Louie, M., Turner, R.J., Howard, C., 2013. Multi-species biofilms defined from drinking water microorganisms provide increased protection against chlorine disinfection. *Biofouling*, 29(8), pp.917–928.

Shakeri, S., Kermanshahi, R., Moghaddam, M.M., Emtiazi, G., 2007. Assessment of biofilm cell removal and killing and biocide efficacy using the microtiter plate test. *Biofouling*, 23(1-2), pp.79–86.

Simões, L.C., 2013. Biofilms in drinking water. In M. Simões & F. Mergulhão, eds. *Biofilms in Bioengineering*. Nova Science Publishers, Inc.

Simões, L.C., Simões, M., 2013. Biofilms in drinking water: problems and solutions. *RSC Advances*, 3(8), pp.2520–2533.

Simões, L.C., Simões, M., Oliveira, R., Vieira, M.J., 2007a. Potential of the adhesion of bacteria isolated from drinking water to materials. *Journal of Basic Microbiology*, 47(2), pp.174–183.

Simões, L.C., Simões, M., Vieira, M.J., 2007b. Biofilm interactions between distinct bacterial genera isolated from drinking water. *Applied and Environmental Microbiology*, 73(19), pp.6192–6200.

Simões, L.C., Simões, M., Vieira, M.J., 2010a. Adhesion and biofilm formation on polystyrene by drinking water-isolated bacteria. *Antonie van Leeuwenhoek*, 98(3), pp.317–329.

Simões, L.C., Simões, M., Vieira, M.J., 2010b. Influence of the diversity of bacterial isolates from drinking water on resistance of biofilms to disinfection. *Applied and Environmental Microbiology*, 76(19), pp.6673–6679.

Simões, M., Simões, L.C., Vieira, M.J., 2009. Species association increases biofilm resistance to chemical and mechanical treatments. *Water Research*, 43(1), pp.229–237.

Simões, M., Simões, L.C., Vieira, M.J., 2010. A review of current and emergent biofilm control strategies. *LWT - Food Science and Technology*, 43(4), pp.573–583.

Siqueira, V.M., 2011. Characterising filamentous fungal biofilm in drinking water distribution systems using microscopic and molecular techniques. PhD thesis, Universidade do Minho.

Siqueira, V., Lima, N., 2012. Surface hydrophobicity of culture and water biofilm of Penicillium spp. *Current Microbiology*, 64(2), pp.93–99.

Siqueira, V.M., Lima, N., 2013. Biofilm Formation by Filamentous Fungi Recovered from a Water System. *Journal of Mycology*, 2013, pp.1–9.

Siqueira, V.M., Oliveira, H.M.B., Santos, C., Paterson, R.R.M., Gusmão, N.B., Lima, N., 2011. Filamentous fungi in drinking water, particularly in relation to biofilm

formation. *International Journal of Environmental Research and Public Health*, 8(2), pp.456–469.

Smits, T.H.M., Wick, L.Y., Harms, H., Keel, C., 2003. Characterization of the surface hydrophobicity of filamentous fungi. *Environmental Microbiology*, 5(2), pp.85–91.

Stepanović, S., Vuković, D., Dakić, I., Savić, B., Švabić-Vlahović, M., 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*, 40(2), pp.175–179.

Szewzyk, U., Szewzyk, R., Manz, W., Schleifer, K.H., 2000. Microbiological safety of drinking water. *Annual Review of Microbiology*, 54, pp.81–127.

Tarkka, M.T., Sarniguet, A., Frey-Klett, P., 2009. Inter-kingdom encounters: recent advances in molecular bacterium-fungus interactions. *Current Genetics*, 55(3), pp.233–243.

Thein, Z.M., Samaranayake, Y.H., Samaranayake, L.P., 2006. Effect of oral bacteria on growth and survival of *Candida albicans* biofilms. *Archives of Oral Biology*, 51(8), pp.672–680.

Verstraeten, N., Braeken, K., Debkumari, B., Fauvart, M., Fransaer, J., Vermant, J., Michiels, J., 2008. Living on a surface: swarming and biofilm formation. *Trends in Microbiology*, 16(10), pp.496–506.

Wargo, M.J., Hogan, D.A., 2006. Fungal-bacterial interactions: a mixed bag of mingling microbes. *Current Opinion in Microbiology*, 9(4), pp.359–364.

WHO, 2011. Guidelines for drinking-water quality - 4th ed. *World Health Organization*.

Wingender, J., Flemming, H.C., 2004. Contamination potential of drinking water distribution network biofilms. *Water Science and Technology*, 49(11-12), pp.277–286.

Yamaguchi, M.U., Rampazzo, R.C.P., Ogatta, S.F.Y., Nakamura, C.V., Ueda-Nakamura, T., Filho, B.P.D., 2007. Yeasts and filamentous fungi in bottled mineral water and tap water from municipal supplies. *Brazilian Archives of Biology and Technology*, 50, pp.1–9.

Yu, J., Kim, D., Lee, T., 2010. Microbial diversity in biofilms on water distribution pipes of different materials. *Water Science and Technology*, 61(1), pp.163–171.

Zhou, L., Zhang, Y., Li, G., 2009. Effect of pipe material and low level disinfectants on biofilm development in a simulated drinking water distribution system. *Journal of Zhejiang University Science A*, 10(5), pp.725–731.

Appendix

Appendix A

In this appendix are presented the values obtained for the contact angles measured with water, formamida and α- bromonaphtalene, as well as the surface tension parameters, the hydrophobicity of microorganisms when immersed in water and free energy of adhesion.

Table A.1- Contact angles (in degrees) with water ($\boldsymbol{\theta_w}$), formamida ($\boldsymbol{\theta_F}$), α- bromonaphtalene ($\boldsymbol{\theta_B}$), surface tension parameters (γ^{lw}- Lifshitz- van der Waals component; γi^+- electron acceptor parameter; γi^-- electron donor parameter), hydrophobicity of microorganisms (i) when immersed in water (w$\Delta \boldsymbol{G_{iwi}^{TOT}}$) () and free energy of adhesion ($\Delta \boldsymbol{G_{1w2}^{TOT}}$) between the microorganisms (1) and PS (2) in water (w) The results presented are means ± SDs of three independent experiments

	Contact Angles (°)			**Surface tension parameters (mJ/m^2)**			**Hydrophobicity (mJ/m^2)**	**Free energy of adhesion (mJ/m^2)**
Bacteria	$\boldsymbol{\theta_w}$	$\boldsymbol{\theta_F}$	$\boldsymbol{\theta_B}$	γ^{LW}	γ^+	γ^-	ΔG_{iwi}^{TOT}	$\Delta \boldsymbol{G_{1w2}^{TOT}}$
A. calcoaceticus	27.54± 1.63	58.82± 1.81	21.62± 4.35	25.57	5.19	44.20	17.42	4.40
Fungal spores								
P. expansum	51.30± 5.49	19.88± 3.28	51.02± 7.93	41.79	0.00	37.41	15.10	-14.10
P. brevicompactum	56± 10.02	20.39± 2.46	42.05± 7.49	41.66	0.22	23.05	-10.92	-25.60
Surface								
PS	83 ± 3.00	71± 2.00	28± 1.00	39.00	0.00	9.90	-44.00	

Appendix B

In this appendix section is presented the image of the water drop after touching the centre of the fungal colony of *P. expansum* grown in MEA, during the surface hydrophobicity measurements.

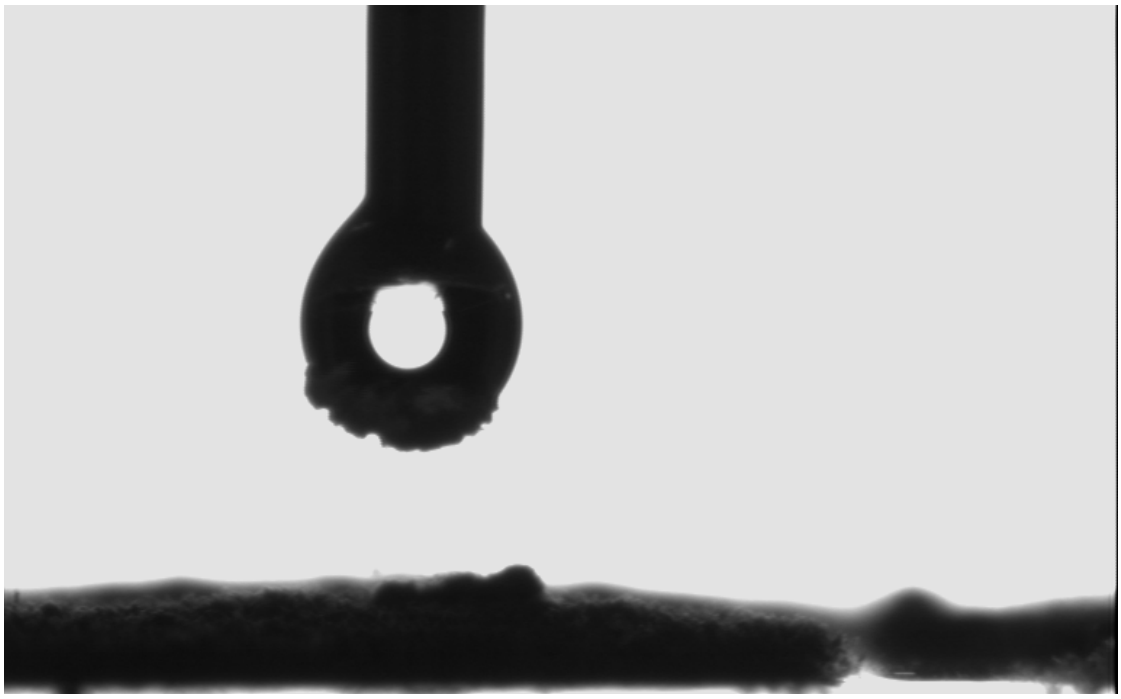

Figure B.1- Water drop with fungal spores after touching the centre region of a fungal colony of *P. expansum* grown in MEA.

Appendix C

In this appendix are presented the mycelial mats on solid culture obtained when *P. expansum* is grown using R2A.

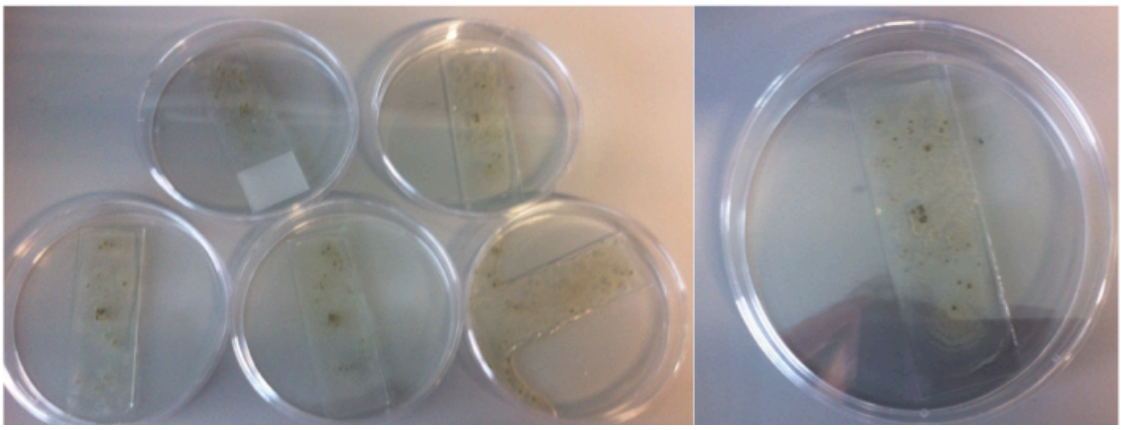

Figure C.1- Mycelial mats of *P. expansum* obtained when the fungi is grown using R2A.

Appendix D

In this Appendix are presented the tables containing the values attained for metabolic activity and biomass quantification, as well as the results calculated for biofilm inactivation and biofilm mass removal percentages for both single and dual-species biofilms after the disinfection treatment using SHC. The results presented for metabolic activity and biofilm mass quantification are means ± SDs of three independent experiments.

Table D.1- Results of the percentage of biofilm inactivation for the control of single and dual-species biofilms at 150 rpm with several SHC concentrations. The values with * were not calculated/considered since the metabolic activity values are higher or equal to the control (0 mg/L)

SHC (mg/L)	***P. expansum***		***P. brevicompactum***		***A. calcoaceticus***		***P. expansum-A. calcoaceticus***		***P. brevicompactum-A. calcoaceticus***	
	Metabolic Activity	Biofilm inactivation (%)	Metabolic Activity	Biofilm inactivation (%)	Metabolic Activity	Biofilm inactivation (%)	Metabolic Activity	Biofilm inactivation (%)	Metabolic Activity	Biofilm inactivation (%)
0	7105 ± 1523		7233 ± 1349		9475 ± 449.90		7939 ± 845.20		7023± 380.10	
0.1	5571± 1146	**22%**	6586 ± 439.50	**9%**	9322 ± 672.70	**2%**	7443± 1467	**6%**	6857± 408.40	**2%**
0.5	3334± 472.10	**53%**	6845 ± 411.70	**5%**	8006 ±1485	**16%**	7861± 639.50	**1%**	7175± 244	**0% ***
1	4447± 933.20	**37%**	7001 ± 459.40	**3%**	5250 ±1808	**45%**	7529± 437.20	**5%**	7139± 354.70	**0% ***
10	3776± 707.90	**47%**	7186 ± 556.90	**1%**	347.10 ± 41.29	**96%**	7347± 558.30	**7%**	5554± 265.20	**21%**
100	5307 ± 466.80	**25%**	7575 ± 649.10	**0% ***	293.60 ± 13.04	**97%**	3638± 790.80	**54%**	583.30± 135.10	**92%**

Table D.2- Results of the percentage of biofilm mass removal for the control of single and dual-species biofilms at 150 rpm with several SHC concentrations

SHC (mg/L)	*P. expansum*		*P. brevicompactum*		*A. calcoaceticus*		*P. expansum-A. calcoaceticus*		*P. brevicompactum-A. calcoaceticus*	
	Biomass quantification	Biofilm mass removal (%)	Biomass quantification	Biofilm mass removal (%)	Biomass quantification	Biofilm mass removal (%)	Biomass quantification	Biofilm mass removal (%)	Biomass quantification	Biofilm mass removal (%)
0	8.90 ± 7.01		7.41± 1.38		1.16± 0.10		10.57± 2.26		10.25± 1.81	
0.1	7.01± 1.56	**22%**	7.08± 1.25	**5%**	0.79± 0.16	**37%**	7.07± 1.46	**34%**	10.10± 2.49	**1%**
0.5	6.82± 1.25	**24%**	6.56± 0.32	**12%**	0.79± 0.20	**36%**	7.20± 2.03	**32%**	9.15± 2.30	**11%**
1	6.04± 0.92	**33%**	7.33± 1.27	**1%**	0.78± 0.20	**38%**	6.35± 1.45	**41%**	8.95± 2.16	**13%**
10	5.74± 0.81	**36%**	6.52± 0.76	**12%**	0.51± 0.12	**66%**	5.68± 1.23	**47%**	6.81± 1.60	**34%**
100	5.47± 1.12	**39%**	6.30± 0.67	**15%**	0.42± 0.10	**74%**	9.92± 1.63	**6%**	6.14± 1.51	**41%**

Table D.3- Results of percentage of biofilm inactivation for the control of single and dual-species biofilms at 25 rpm with several SCH concentrations. The values with * were not calculated/considered since the metabolic activity values are higher or equal to the control (0 mg/L)

SHC (mg/L)	*P. expansum*		*P. brevicompactum*		*A. calcoaceticus*		*P. expansum-A. calcoaceticus*		*P. brevicompactum-A. calcoaceticus*	
	Metabolic Activity	Biofilm inactivation (%)	Metabolic Activity	Biofilm inactivation (%)	Metabolic Activity	Biofilm inactivation (%)	Metabolic Activity	Biofilm inactivation (%)	Metabolic Activity	Biofilm inactivation (%)
0	4108 ± 1216		7201± 182.30		5423± 1500		9810±748.30		7330± 204	
0.1	2026± 502.10	**51%**	7220± 706.80	**0% ***	4484± 823.90	**17%**	9026±2161	**8%**	7344± 200.60	**0% ***
0.5	1656± 402.90	**60%**	7321± 232.20	**0% ***	4448± 899.30	**18%**	8534± 1297	**13%**	7344± 240.60	**0% ***
1	1459± 543.40	**64%**	7130± 492.60	**1%**	3877± 940.20	**29%**	8516± 875	**13%**	7360± 193.70	**0% ***
10	389.5± 171.90	**91%**	7585± 358.20	**0% ***	368.50± 12.32	**93%**	5784±694.70	**41%**	6314± 744.90	**14%**
100	2211± 390.90	**46%**	7282± 695.50	**0% ***	357.50± 11.47	**93%**	415.9± 21.47	**96%**	303.20± 31.95	**96%**

Table D.4- Results of the percentage of biofilm mass removal for the control of single and dual-species biofilms at 25 rpm with several SHC concentrations

SHC (mg/L)	*P. expansum*		*P. brevicompactum*		*A. calcoaceticus*		*P. expansum-A. calcoaceticus*		*P. brevicompactum-A. calcoaceticus*	
	Biomass quantification	Biofilm mass removal (%)	Biomass quantification	Biofilm mass removal (%)	Biomass quantification	Biofilm mass removal (%)	Biomass quantification	Biofilm mass removal (%)	Biomass quantification	Biofilm mass removal (%)
0	7.20± 1.37		4.01± 1.00		0.81± 0.21		2.30± 0.18		3.74± 0.78	
0.1	5.07± 0.67	**30%**	4.85± 0.86	**0% ***	0.62± 0.16	**29%**	1.86± 0.45	**21%**	3.11± 0.79	**18%**
0.5	4.77± 0.77	**35%**	5.27± 0.93	**0% ***	0.62± 0.15	**30%**	1.39± 0.29	**43%**	3. 93 ± 0.82	**0% ***
1	4.29± 0.76	**41%**	5.90± 0.78	**0% ***	0.69± 0.17	**19%**	2.24± 0.33	**3%**	3.74 ± 0.94	**0% ***
10	4.07± 0.67	**44%**	8.87± 3.22	**0% ***	0.42± 0.10	**60%**	3.20 ± 0.45	**0% ***	3.46± 0.64	**8%**
100	4.70± 0.81	**36%**	6.32± 1.87	**0% ***	0.31± 0.08	**77%**	5.49 ± 0.84	**0% ***	3.63 ± 0.91	**3%**